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A multifactorial approach to the biological role of the mycobacterial maltokinase

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Ana Branco Maranha Tiago

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Abbreviations utilized

TB – Human tuberculosis

MDR-TB - Multiple-Drug-Resistant Tuberculosis

XDR-TB – Extensively-Drug Resistant Tuberculosis

FAS - Fatty Acid Synthetase

TDM - trehalose-dimycolate

TMM - trehalose-monomycolate

T6P - treahalose-6-phosphate

MGLP - Methylglucose lipopolysaccharide

OtsAB - Osmotically-regulated trehalose synthesis

Tps or OtsA - Trehalose 6-phosphate synthase

Tpp or OtsB - Trehalose 6-phosphate phosphatase

TreY - Maltooligosyltrehalose synthase

TreZ - Maltooligosyltrehalose hydrolase

TreS - Trehalose synthase

MTase – TreS trehalose interconverting activity

GMPMT or GlgE - α1,4-glucan: maltose-1-phosphate maltosyl-transferase

Mal1P – Maltose-1-phosphate

Mak - Maltokinase

APHs - Aminoglycoside phosphotransferases

TLC – Thin Layer Chromatography

SDS-PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

RT-PCR – Real-Time Polymerase Chain Reaction

Abstract

Twenty years ago Human tuberculosis (TB) was thought to be a disease on the verge of eradication. Nowadays TB is one of the deadliest diseases in the world and the WHO estimates that more than one-third of the world's population is infected with TB.

Maltokinase (Mak) is an enzyme essential for mycobacterial growth that is present in nearly all mycobacterial genomes. Nonetheless the physiological role of this enzyme remains to be fully elucidated.

In an effort towards unveiling the role of Mak in mycobacterial physiology additional functions of the enzyme *in vitro* were explored, namely by testing possible alternative substrates (phosphoryl acceptors) as well as the possibility of the involvement of this enzyme in the inactivation of aminoglycoside antibiotics. A preliminary analysis of the transcriptional responses of the corresponding gene in *M. smegmatis*, in different nutritional conditions, was attempted. Concurrently, to confirm the proposed essentiality of Mak, *M. marinum* was selected as the tool for the construction of a *mak* conditional knock-out mutant, and thus, evaluate the effects of silencing the gene on the mutant's phenotype.

Recombinant His-tagged Mak was purified to further test substrate specificity. The purification protocol was made less laborious by the modification of the sodium phosphate buffer pH. Subsequently several substrates were tested as phosphoryl group acceptors Namely aminoglycoside antibiotics, disaccharides and trisaccharides, however no activity was detected, except for maltose, implicating that in *M. smegmatis*

the role of phosphate acceptor might be specific to maltose. It is then extremely unlikely the protein could render aminoglycoside resistance to bacteria.

To evaluate the relation between *mak* expression and the nutritional source in the growth medium the activity of the enzyme was determined in *M. smegmatis* cell-free extracts. The results indicate that Mak activity is significantly affected by the presence of maltose in the medium. In order to analyze the transcriptional responses of the *mak* gene in different nutritional conditions an RT-PCR experiment was optimized but due to technical problems and time constraints, no further progress could be made beyond this stage.

The design and construction for the engineering of the *mak* conditional mutant done in this work will, in the future, allow the conditional expression of genes essential for mycobacterial growth and in doing so, provide a better understanding of their involvement in mycobacterial metabolism.

Keywords: maltokinase, maltose-1-phosphate, mycobacteria, metabolism, RT-PCR.

Resumo

Há cerca de vinte anos pensava-se que a tuberculose era uma doença em vias de erradicação no entanto, hoje em dia, é uma das doenças mais mortíferas do mundo. A Organização Mundial de Saúde estima que mais de um terço da população mundial esteja infectada com tuberculose.

A maltocinase (Mak) é uma enzima essencial para o crescimento micobacteriano que se encontra presente na maioria dos genomas micobacterianos anotados. Contudo, o papel fisiológico desta enzima ainda não é totalmente conhecido.

Numa tentativa de elucidar o papel desempenhado pela Mak na fisiologia micobacteriana foram, neste trabalho, exploradas funções adicionais da proteína, *in vitro*. Foram testados substratos alternativos (aceitadores de grupo fosforil), bem como a possibilidade de a enzima estar envolvida na inactivação de antibióticos aminoglicosídeos. Foi ainda realizado um ensaio preliminar para análise das respostas transcripcionais do gene correspondente em *M. smegmatis*, em diferentes condições nutricionais. Simultaneamente, para confirmar o papel essencial proposto para a Mak, o organismo *M. marinum* foi seleccionado, como ferramenta para construção de um mutante knock-out condicional para *mak*, para deste modo avaliar os efeitos do silenciamento do gene no fenótipo do mutante.

A proteína recombinante, com cauda de histidinas, foi purificada com vista a serem efectuados testes de especificidade de substratos. O protocolo de purificação foi modificado, através da alteração do pH do tampão fosfato de sódio, de modo a encurtar a duração da purificação. Em seguida, vários substratos foram testados como

aceitadores de grupos fosforil, nomeadamente antibióticos aminoglicosídeos, dissacarídeos e trissacarídeos. No entanto, não foi detectada qualquer actividade, excepto para a maltose, o que implica que em *M. smegmatis* o papel de aceitador de grupos fosfatos deve ser específico para este açúcar. É pois extremamente improvável que a proteína Mak confira resistência a aminoglicosídeos.

Para avaliar a relação entre a expressão do gene *mak* de *M. smegmatis* e diferentes fontes de carbono no meio de cultura, determinámos a actividade da enzima em extractos celulares de *M. smegmatis*. Os resultados indicam que a actividade da Mak é significativamente afectada pela presença de maltose no meio. Com o objectivo de analisar as respostas trascripcionais do gene *mak* em diferentes condições nutricionais uma experiência de RT-PCR foi optimizada. No entanto, devido a uma série de problemas de ordem técnica e limitações a nível temporal, não foi possível progredir para além do que é apresentado neste trabalho.

O desenho e construção para a criação do mutante condicional para a *mak* apresentado neste trabalho vão permitir, futuramente, a expressão condicional de genes essenciais ao crescimento micobacteriano, permitindo deste modo um melhor entendimento do seu envolvimento no metabolismo destas bactérias.

Palavras-chave: maltocinase, maltose-1-fosfato, micobactéria, metabolismo, RT-PCR

Chapter I Introduction

1. Tuberculosis

1.1. Tuberculosis' Occurrence

Human tuberculosis (TB) is one of the deadliest diseases in the world despite the multitude of strategies to prevent and control the disease. The World Health Organization (WHO) estimates more than one-third of the world's population to be infected with TB (first contact). In 2008 this disease took more than 1.8 million lives (WHO report 2009). TB is also one of the leading causes of death among HIV infected people.

Twenty years ago TB was thought to be a disease on the verge of eradication. The problem, however, has become more severe. Not only due to the synergy between HIV/AIDS and tuberculosis or the combination of the HIV epidemic with an urban drug problem, but also owing to the lack of drug compliance and subsequent appearance of multiple-drug-resistant strains (MDR-TB), in addition to the inadequacy of access to proper medical care in developing countries. This scenery provided fertile ground on which tuberculosis could flourish anew (Saviola & Bishai 2006; WHO report 2009).

In 2007 there were 9 million TB case notifications, 5 thousand of which were estimated to be new MDR-TB cases (resistance to standard first-line drugs) (TB fact sheet 2009) and recent numbers indicate that XDR-TB cases (resistance to second-line drugs in addition to MDR-TB) comprise 15% of MDR-TB cases (XDR TB fact sheet).

The incidence of atypical mycobacterial infections has also increased in recent years. Opportunistic diseases caused by the *Mycobacterium avium* complex (MAC) were uncommon prior to the onset of the AIDS epidemic. Nowadays they remain a leading cause of mortality among HIV-infected people (Center for Disease Control and

Prevention's (CDC) Guidelines for Prevention Treatment of Opportunistic Infections in HIV-Infected Adults and Adolescents, 2009).

Global efforts to control TB were reinvigorated in 1991, when a World Health Assembly (WHA) resolution recognized TB as a major global public health problem, afterwards in 1993 the WHO declared tuberculosis a global health emergency and since then stopping TB has been an ongoing challenge undertaken by many organizations.

1.2. The Genus *Mycobacterium*

For many years the causative agent of tuberculosis remained a mystery as did the etiology of the disease. In 1868 Jean Antoine Villemin publishes "Etudes sur la tuberculose", the results of the first experimentations in the field, in which he verified that the disease was in fact transmissible. Nonetheless mycobacteria were discovered only a decade later when Robert Koch isolated the *Tubercle bacilli*. The German physician presented his discovery of *Mycobacterium tuberculosis* in a lecture in 1882 and later in a German medical journal the Berliner Medicinische Wochenschrift under the title "Die Aetiologie der Tuberculose". A few years later, Armauer Hansen identified the bacillus responsible for leprosy (Tan & Graham, 2008).

In 1896 a new classification to these organisms, the genus *Mycobacterium,* is proposed by Lehmann and Neumann, it would include these species (http://www.bacterio.cict.fr/m/mycobacterium.html), now renamed *Mycobacterium tuberculosis* and *Mycobacterium leprae.* The organisms were included in the *Mycobacteriaceae* family, sub-order *Corynebacterineae*, order *Actinomycetales* and class *Actinomycetes*. The order *Actinomycetales* is part of a larger group, the Gram-positive bacteria with a high G+C content, or *Actinobacteria*. Within the suborder

Corynebacterineae there are eight groups of great clinical or environmental importance such as *Corynebacterium*, *Nocardia* and *Rhodococcus* (Zhi *et al*., 2009).

Currently, the minimal criteria that allow inclusion in the genus *Mycobacterium* dictate that the species must be acid fast, following Ziehl-Neelsen staining; it must contain mycolic acids of 60–90 carbon atoms in length and its genome must have a G+C content of 61–71%. According to J.P. Euzéby (http://www.bacterio.cict.fr/) there are 148 species of *Mycobacterium* that fit these criteria and are shown to be closely related through 16s ribosomal RNA (rRNA) gene analysis (Tortoli, 2006; Sidders & Stoker, 2007).

Based on their generation or duplication time mycobacteria can be divided into two groups, the slow growing mycobacteria and the fast growing mycobacteria. *M. tuberculosis*, a slow grower, has a generation time, *in vitro*, of about 20 hours (Hiriyanna & Ramakrishnan, 1986); while the generation time for *M. smegmatis*, a fast grower, is *circa* 3 hours (Hiriyanna & Ramakrishnan, 1986). Traditionally, they are distinguished by their ability to produce visible colonies on solid media within 7 days. Fast growing mycobacteria need up to seven days whereas slow growing mycobacteria need more than seven days (Sidders & Stoker, 2007).

Most pathogenic mycobacteria are slow growers, although there are exceptions, such as the *Mycobacterium fortuitum* group, the *M. chelonae/abscessus* group, and the *M. smegmatis* group. These organisms comprise a group of rapidly growing mycobacteria that are found in soil samples as well as in water. Outbreaks are often associated with health care-associated infections and usually occur after a traumatic injury followed by potential soil or water contamination (Brown-Elliot & Wallace Jr, 2002). Furthermore

for immunocompromised individuals even nonpathogenic *Mycobacterium* species may become pathogenic (Shinnick & Good, 1994; Sidders & Stoker, 2007).

The *Mycobacterium avium* complex (MAC) currently includes eight slow growing mycobacterial species. *Mycobacterium avium, M. intracellulare, M. chimaera, M. colombiense, M. arosiense, M. bouchedurhonense, M. marseillense* and *M. timonense*. In humans, as previously stated, these organisms are the cause of opportunistic infections (Cayrou *et al*., 2010).

The mycobacteria that cause human tuberculosis are grouped together in what is known as the *M. tuberculosis* complex: it includes *M. tuberculosis, M. bovis, M. caprae, M. africanum, M. microti, M. pinnipedii* and *M. canettii* (http://www.uniprot.org/taxonomy/77643; Aranaz *et al*., 2003).

Mycobacterium tuberculosis is an obligate pathogen which causes tuberculosis in humans and the most studied of all mycobacteria (Glickman & Jacobs Jr., 2001).

1.2.1. Mycobacterium marinum

In 1926, Aronson isolated and described a mycobacterium responsible for the death of saltwater fish in the Philadelphia aquarium. The pathogen caused a tuberculosis-like disease in fish. He named that organism *Mycobacterium marinum* (Gluckman, 1995).

M. marinum's optimal growth temperature is 25-35 ºC and its generation time is approximately 4.5 hours (Clark & Shepard, 1963). This pathogen is adapted to infect ectotherms. Its low optimal growth temperature most likely explains the limitation of its infection ability to human hosts' extremities. The infection caused by *M. marinum* is named fish tank granuloma or swimming pool granuloma. Pathologically, the

granuloma caused by *M. marinum* dermal infection is similar to that caused by *M. tuberculosis* lung infection (Stamm & Brown, 2004).

M. marinum is photochromogenic, producing a characteristic yellow color when exposed to light. The expression of pigmented molecules like carotenoids is believed to confer resistance to UV damage (Stamm & Brown, 2004).

The pathological similarity of *M. marinum* to *M. tuberculosis* infection combined with its increased safety (biosafety level 2), more rapid growth and easier use, makes *M. marinum* an useful model with which to study pathogenic mycobacteria. *M. marinum* is also widely used to elucidate mycobacterial molecular mechanisms. Despite the obvious utility of the model, it is crucial to remember it carries unique adaptations and a direct comparison with *M. tuberculosis* will reveal both shared and divergent pathways.

Tønjum *et al*. (1997) once having analyzed the 16s rRNA sequences of nineteeen mycobacterial species concluded that *M. marinum*, followed by *M. ulcerans*, are the two mycobacterial species outside the *M. tuberculosis* complex most closely related to *M. tuberculosis.* This result is supported by DNA-DNA hybridization studies. In addition studies conducted by Stinear *et al.* (2008) state that there is a *M. marinum* ortholog for 80% of all coding sequences in *M. tuberculosis*, with an average shared amino acid identity of 85%.

The complete genome sequence of *M. marinum* was determined by Stinear *et al*. (2008). The authors compared the organism's genome with further mycobacterial genomes, among them the *M. tuberculosis* genome. *M. marinum* comprises a 6.6 Mb circular chromosome, the size discrepancy comparatively to *M. tuberculosis'* genome (4.4 Mb Cole *et al.*, 1998) reflects *M. marinum´*s larger host range and environmental niche. The

authors hypothesize that the genome comparison data indicate that *M. marinum* and *M. tuberculosis* have diverged from a common generalist environmental *Mycobacterium* with *M. marinum* retaining genes required for its dual lifestyle and *M. tuberculosis* undergoing reductive evolution, compatible with adaptation to its decreased niche. The same explanation is provided for the fact that whole genome comparisons between *M. tuberculosis, M. marinum* and *M. smegmatis* reveal a higher level of colinearity, synteny, and conservation of chromosome size between *M. marinum* and the distant saprophyte *M. smegmatis* (76% average amino acid identity) than with *M. tuberculosis*.

1.2.2. Mycobacterium smegmatis

In 1884 Lustgarten reported he had found a bacillus similar to the *Tubercle bacilli* in shyphilic chancres and gummae. Subsequently Alvarez and Tavel found the same organisms in normal genital secretions (smegma) of a patient with a penile ulcer (Wallace Jr. *et al*., 1988).

M. smegmatis is altogether a rapidly growing ubiquitous saprophyte and the few related diseases are most commonly encountered in clinical practice. The organism can cause soft tissue infection, post-traumatic wound infections and surgical wound infections. As previously stated these healthcare-associated infections are frequently a result of direct traumatic inoculation of contaminated material (Brown-Elliot & Wallace Jr, 2002).

The study of *M. tuberculosis* using *M. smegmatis* as a model system has some disadvantages. One of its major inconveniences is the evolutionary distance between *M. smegmatis* and *M. tuberculosis* which is far greater than between *M. tuberculosis* and the other commonly utilized models (such as *M. marinum* or *M. bovis*). *M. smegmatis* has a 6.98 Mb genome (http://cmr.jcvi.org/tigr-scripts/CMR/shared/Genomes.cg)

significantly differing from *M. tuberculosis'* genome size (4.4 Mb; Cole *et al.*, 1998) which, as previously explained, reflects its broader environmental niche and saprophytic lifestyle. Furthermore *M. smegmatis* has none of the pathogenic properties described for *M. tuberculosis*. On the other hand, *M. smegmatis* is a particularly appealing model for mycobacterial study because of its rapid growth and ease of genetic manipulation. *M. smegmatis* mc^2155, the most commonly used laboratory strain, is considered a nonpathogenic mutant of the parental strain mc^6. It can be readily transformed with foreign DNA using electroporation, making it extremely useful for expressing mycobacterial genes. Mutations in the *M. smegmatis'* genes can be readily complemented with genes from *M. tuberculosis*, thus proving it can be a valuable surrogate host (Snapper *et al.*, 1990; Gupta & Chatterji, 2005; Shiloh & Champion, 2010).

The conclusion to be drawn from the previous statements is that *M. smegmatis* is an extremely helpful model for comparative biology and genetic studies although it lacks credibility as an infection model.

2. The Importance of Trehalose

Sugars are fundamental to life. Sugars are an immediate and transportable energy source. They can also furnish structural components as well as play a role as chemical messengers conveying information. The structural versatility of carbohydrates provides the potential for a communication system, or glycocode (Paul *et al.*, 2008).

Trehalose is a sugar significant for its protective ability. It is a known compatible solute under osmotic stress, stable and with low reactivity (being a non reducing sugar). It protects proteins and membranes from denaturation by replacing water and during

desiccation trehalose prevents protein aggregation and free radical diffusion. It may even have a role as a signaling molecule coordinating metabolism (Woodruff *et al*., 2004; Pan *et al*., 2004; Paul *et al*., 2008).

Trehalose is an ubiquitous glucose disaccharide prevalent in mycobacteria, not only in the free form in the cytoplasm but also as a basic component of a number of cell wall glycolipids (Elbein *et al*., 2003).

A distinctive characteristic of the *Mycobacteriaceae* family closely related with its pathogenic capability is its unique cell wall. The mycobacterial cell wall consists of an inner layer and an outer layer. The inner compartment comprises peptidoglycan (PG), arabinogalactan (AG) and mycolic acids (mycolic acids are β-hydroxy fatty acids with a long α-alkyl side chain (*cf.* Fig. 1)) covalently linked to form a complex that extends from the plasma membrane outward in layers (*cf.* Fig. 2). This insoluble complex causes the bacteria to be impervious to a great number of chemical compounds and is in part responsible for its resistance to most common antibiotics (Cole *et al*. 1998; Sidders & Stoker, 2007; Hett & Rubin, 2008). The outer layer, or capsule, is composed by carbohydrates, proteins and lipids, organized as a bilayer structure. These outer proteins and lipids are soluble components of the cell wall and have been referred to as the signaling and effector molecules of mycobacteria because of their known roles in interacting with the immune system (Hett & Rubin, 2008; Hoffmann *et al*., 2008).

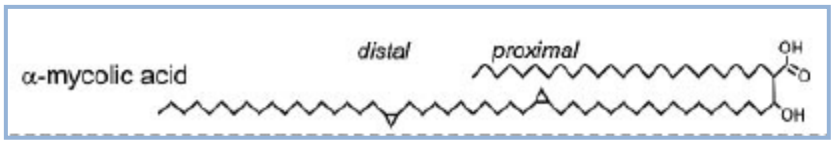


Figure 1 - Chemical structures of mycolic acid from *M. tuberculosis*. Adapted from Takayama *et al.* (2005)

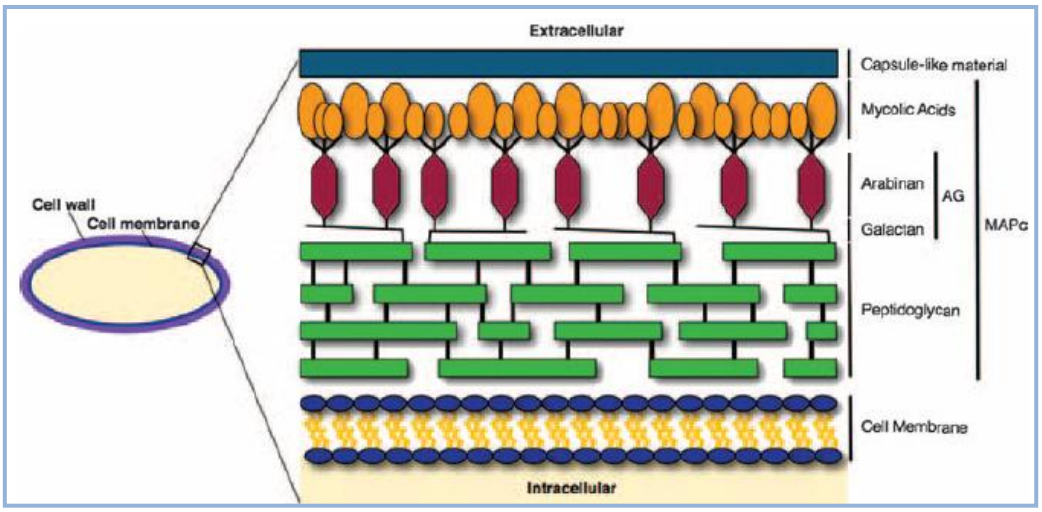


Figure 2 - Schematic representation of the basic components of the mycobacterial cell wall. Adapted from Hett & Rubin (2008)

The most common cell wall trehalose lipid is cord factor, or trehalose-dimycolate (TDM). This lipid is considered to be closely associated with mycobacterial toxicity (Elbein *et al.*, 2003).

2.1. Mycolic Acids

In mycobacteria Fatty Acid Synthetase I and II (FAS) are multi-enzyme complexes involved in mycolic acid biosynthesis (Takayama *et al.*, 2005).

Once the mycolic acid is synthesized it needs to be processed. This three step process consists of the transport of the mycolate outside the cell and linkage to the PG-AG complex, to yield TDM. The first reaction, transfer of the mycolyl group to treahalose-6-phosphate (T6P) to yield TMM-phosphate (trehalose-monomycolate-phosphate), is followed by dephosphorylation of this product. The outcome, TMM, is then transported

outside the cell outer membrane where it is involved in the synthesis of both TDM and cell wall arabinogalactanmycolate (Takayama *et al*., 2005).

Mycobacteria produce two unusual polymethylated polysaccharides (PMPS) the methylmannose polysaccharide (MMP) and the methylglucose lipopolysaccharide (MGLP). Both polysaccharides are thought to regulate fatty acid synthesis by FAS-I as a consequence of their ability to form stable complexes with fatty acids and acyl coenzyme. Among other functions PMPS were also proposed to serve as general lipid carriers facilitating the synthesis of the very large and insoluble mycolic acid esters (Jackson & Brennan, 2009).

The advantage of forming a TMM-phosphate intermediate rather than TMM directly from trehalose is not clear. Although being somewhat controversial, studies demonstrate that only T6P, but not trehalose, can stimulate mycolate production (Shimakata & Minatogawa, 2000) anticipating a possible role as a signaling molecule for T6P. Also a contentious issue is the final steps of mycolate biosynthesis.

Tropis *et al.* (2005) contradict this model suggesting that mycolic acid biosynthesis takes place in cellular compartments other than the cytoplasm. The evidence supporting this statement is that *Corynebacterium glutamicum* mutants lacking the ability to produce trehalose could produce TMM, after being rescued with extrinsic trehalose and, most importantly, the sugar was not transported inside the cell (verified by radio labeling). The authors also constructed knock-out mutants for the synthesis of T6P and did not find differences in mycolate content, when compared to bacteria capable of synthesizing T6P, which lead them to state that T6P has no significant role in mycolic acid synthesis.

2.2. Biosynthetic Pathways

Most mycobacteria have three trehalose biosynthetic pathways, *M. tuberculosis, M. marinum* and *M. smegmatis* are no exception (De Smet *et al*., 2000; Woodruff *et al.*, 2004; Tropis *et al.,* 2005). The same three pathways were also found in *C. glutamicum* genome. However, while in *M. smegmatis* (Woodruff et al., 2004) and *M. tuberculosis* (Murphy *et al*., 2005) trehalose synthesis is essential for bacterial growth, the same is not true for *Corynebacterium.* In these organisms it is possible to construct viable knock-out mutants for all three biosynthetic pathways (Tropis *et al.*, 2005).

Trehalose biosynthetic pathways are named after the corresponding genes OtsAB (or Tps/Tpp), TreYZ and TreS.

The OtsAB pathway catalyzes the transfer of glucose from UDP-glucose to glucose-6-phosphate (glucose 6P) to produce UDP plus T6P, which is subsequently dephosphorylated into trehalose. The reactions are catalyzed by trehalose 6-phosphate synthase (Tps or OtsA) and trehalose 6-phosphate phosphatase (Tpp or OtsB) (*cf.* Fig. 3), in *E. coli* these enzymes are called OtsA and OtsB (osmotically-regulated trehalose synthesis) (De Smet *et al*., 2000; Elbein *et al*., 2003; Tropis *et al*., 2005).

The TreYZ pathway for trehalose synthesis uses oligo/polymaltooligodextrins or glycogen as substrate. The terminal α -1,4 glycosidic bond at the reducing end of the polymer is converted into α -1,1 by maltooligosyltrehalose synthase (TreY) and the resulting terminal trehalosyl unit of the polysaccharide is then released by maltooligosyltrehalose hydrolase (TreZ) (*cf.* Fig. 3)(De Smet *et al*., 2000; Elbein *et al*., 2003; Tropis *et al*., 2005).

The TreS pathway is able to interconvert maltose into trehalose. The reaction is catalyzed by trehalose synthase (TreS), which converts the α -1,4 glycosidic bond of maltose into an α -1,1 linkage to yield trehalose. TreS is also capable of functioning in the reverse direction although (*cf.* Fig. 3) it shows more affinity to the conversion of maltose to trehalose (maltose Km=10 mM; trehalose Km=90 mM) (Pan *et al*., 2004).

Pan *et al*. (2008) suggest that TreS has two different active sites. One is responsible for the maltose trehalose interconverting activity (MTase) (Pan *et al*., 2004) and the other for the amylase activity, which can produce maltose from either glycogen or maltooligosaccharides (*cf.* Fig. 3) (demonstrated in the former paper). The authors propose that TreS plays a key role in the interaction between trehalose and glycogen, when cytoplasmic trehalose levels are low, by catalyzing the conversion of glycogen to maltose and maltose to trehalose. Interestingly, the authors observed that high levels of trehalose in the cytoplasm also appeared to cause or stimulate the accumulation of glycogen in these cells. Subsequently Elbein *et al.* (2010) partially purified the enzyme GMPMT (α1,4-glucan: maltose-1-phosphate maltosyl-transferase) (or GlgE Kalscheuer *et al*., 2010) which is involved in the last steps of the conversion of trehalose to glycogen. The authors hypothesize that the enzyme might also function as a sensor or regulator of trehalose levels within the cell. As such, TreS can expedite the conversion of excess trehalose to glycogen when trehalose levels become dangerously high. From an energetic perspective this would be the most viable option. Although cells can convert trehalose to glucose, glucose can not be stored and energy would be lost. In stressful situations, for example after heat stress, trehalose levels would be high and ATP levels would likely be below inhibitory concentration (GMPMT is inhibited by physiological levels of ATP). Under these conditions, the maltosyl transferase pathway

would be activated and the excess trehalose could be converted to glycogen and stored for future use. Thus, the authors postulate that the major role of this pathway may be to convert excess trehalose to glycogen (*cf.* Fig. 3).

Woodruff et al. (2004) constructed a mutant strain of *M. smegmatis* lacking all three trehalose biosynthesis pathways. They demonstrated that even though trehalose is vital for growth, the three pathways are functionally redundant, and possibly can substitute one another.

Murphy *et al.* (2005) introduced mutations into each of the three trehalose biosynthesis pathways in *M. tuberculosis* and established a clear dominance of the Tps/Tpp pathway. The authors also concluded that TreYZ pathway is not essential for *M. tuberculosis'* growth. A knock out mutant for Tpp2 (*M. tuberculosis* has two *tpp* genes) displayed a lethal phenotype and could not, conversely to other mutants defective for trehalose synthesis, be rescued by exogenous trehalose. Severe growth defects presented by the mutant demonstrate that trehalose phosphatase plays an essential role that cannot be accomplished by any other enzyme. The lack of phenotype for the Tps1 knock out clone is consistent with both the previous remark and the observation made by the authors that the protein product of Tps1 may lack functional trehalose-6-phosphate phosphatase activity. The Tpp2 mutant accumulates T6P which can be detrimental to the organism (Paul *et al.*, 2008). According to Murphy *et al.* (2005) the lethal phenotype indicates that T6P has in *Mycobacterium* a signaling role similar to the one it plays in yeast.

Mutant strains lacking TreS exhibited impaired infection capability, *in vivo*, which could reflect a need for the interconversion of stored trehalose into maltose and then

into usable glucose, during persistent infection with *M. tuberculosis* (Murphy *et al.*, 2005).

Tropis *et al.* (2005) constructed mutant strains of *Corynebacterium glutamicum* unable to synthesize trehalose due to the knock-out of the genes for all three pathways of trehalose biosynthesis. The inability of the mutants to synthesize trehalose, in addition to an increase in osmosensitivity, led the cells to exhibit a stronger aggregation in liquid culture. After centrifugation of the cultures, analysis of the supernatant of the triple mutant strain revealed that the protein concentration was roughly 7-fold higher than in the wild-type. This suggests that trehalose might have an important role in protein stabilization.

The authors also concluded that in the absence of trehalose *C. glutamicum* was capable of synthesizing mycolates using other sugars, such as glucose, maltose or maltotriose, as acceptors. Thus, in principle, the only requirement for mycolate synthesis would be the presence of an α-glucosyl-containing sugar in the medium. Hence, contradicting the general conviction that trehalose plays an essential role as a mycolate acceptor and in the resulting production of TMM; the authors consider the role of trehalose not to be a prerequisite for cell wall linked mycolate synthesis.

3. Maltose and Maltose-1-phosphate

Disaccharides are popular carbon sources which can be consumed, generally after breakdown to monosaccharides, followed by ATP dependent phosphorylation and subsequent incorporation into the central catabolic pathways. In bacterial sugar metabolism, the ATP-dependent phosphorylation of disaccharides does not seem to play

a significant role, it seems however to be widespread during transport (Postma *et al*., 1993; Deutcher *et al*., 2006).

Phosphorylation of disaccharides in the cytoplasm prior to degradation is a rare phenomenon which was identified in *Aerobacter aerogenes* in 1972 (Palmer & Anderson, 1972 a). This organism phosphorylates cellobiose in position 6 with consumption of ATP prior to hydrolysis (Palmer& Anderson, 1972 b). Latter, in 1996, Drepper *et al*. identified an enzyme (Maltokinase) capable of catalyzing an ATP-dependent phosphorylation of maltose to maltose-1-phosphate, in cell-free extract of *Actinoplanes sp*.

It was also reported that *E. coli* seems to be able to produce maltose-1-phosphate (mal1P) (Decker *et al,*. 1999) via TreC in an ATP-independent manner. The authors speculated that the phosphorylated disaccharide might play a role as an inducer of the *mal* operon.

Although the *glgE* gene and its homolog were considered essential for the growth of *M. tuberculosis* (Sassetti *et al*., 2003) and *M. smegmatis,* Kalscheuer *et al*. (2010) were able to generate null deletion mutations of the gene in *M. smegmatis*, in minimal medium. The authors observed intriguing modifications in the Δ*glgE* mutants. The mutants accumulated large amounts of mal1P and were sensitive to the disaccharide trehalose, which induced bacteriostasis correlating with mal1P hyperaccumulation. Second-site gene deletions in the *treS* and *pep2* (*mak*) genes conferred trehalose resistance and suppressed mal1P accumulation, caused by *glgE* inactivation. Inactivation of *treS* or *pep2* genes prevented mal1P formation altogether in the Δ*glgE* mutant. Substantial

levels of the radiolabeled maltose intermediate were detectable only in Δ*pep2* strains, indicating its fast turnover to mal1P by Pep2 (Mak).

The *glgB* gene, which was demonstrated to be essential in *M. tuberculosis* H37Rv, clusters with *glgE* and encodes a branching enzyme that is required for introducing α-1,6-linked branches into linear α-1,4-glucans , for example glycogen. The authors found *glgB* to be nonessential in *M. smegmatis*. The *M. smegmatis* Δ*glgB* mutant was likewise sensitive to trehalose and trehalose sensitivity in an *M. smegmatis* Δ*glgB* strain could again be suppressed by deletion of the *treS* gene.

GlgE inactivation is lethal in *M. tuberculosis* and the authors were unsuccessful in deleting *glgE* in the wild-type, but the gene could readily be inactivated in the Δ*treS* mutant, confirming both the essentiality of GlgE and the causality of mal1P toxicity, respectively. Likewise, *treS* inactivation allowed deletion of *glgB*, demonstrating that mal1P toxicity is also the probable cause of GlgB essentiality.

Cytoplasmic mal1P accumulation in the *M. smegmatis* Δ*glgE* mutant suggested that the phosphorilated sugar might be the direct substrate of GlgE. Also the unveiling of the mal1P-dependent maltosyltransferase activity of GlgE, combined with the phenotypes of the *M. smegmatis* mutants, allowed the authors to conclude that GlgE is part of a pathway that converts trehalose into an α-1,6-branched α-1,4-glucans via four enzymatic steps mediated by TreS, Pep2 (Mak), GlgE and GlgB. GlgE not only forms the linear α-1,4-glucan but it can also edit the branch lengths of branched glucan (*cf.* Fig. 3).

The authors found that mal1P accumulation, as a result of GlgE inactivation, appeared to elicit pleiotropic stress responses in *M. tuberculosis*, including inhibition of

respiration and induction of the stringent response as well as DNA damage (Kalscheuer *et al*., 2010). In the authors opinion the mal1P-induced lethality might share certain aspects of the common mechanism of cellular death caused by bactericidal antibiotics in *E. coli*. However the authors do not exclude the hypothesis that mal1P might not be directly toxic to the cells but could rather lead indirectly to toxicity by more complex mechanisms.

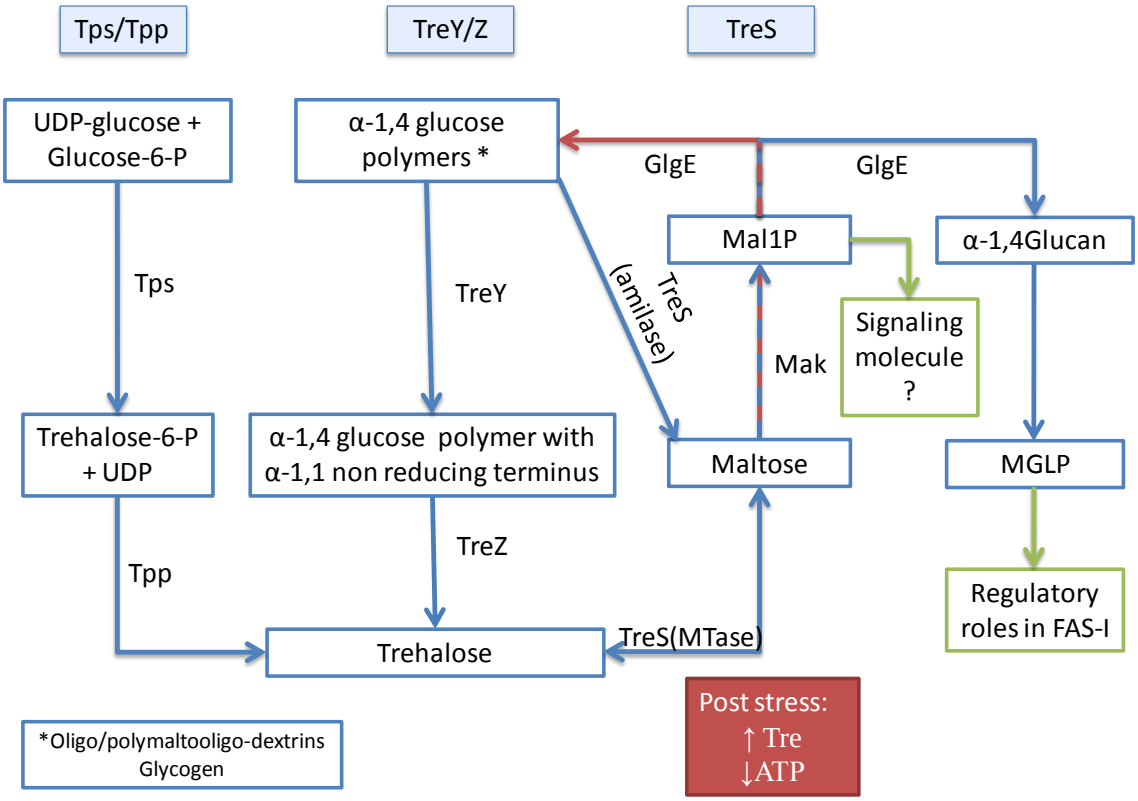


Figure 3 – Summary representation of the pathways related to maltose-1-phosphate. Adapted from Elbein *et al*. (2003); Tropis *et al*. (2005); Pan *et al*. (2004); Pan *et al*. (2008); Elbein *et al*. (2010) and Kalscheuer *et al*. (2010)

In the early 90's it was discovered that, in yeast, the trehalose synthase small subunit, (TSS1), as well as synthesizing T6P, controlled glucose influx into glycolysis. One of the hypotheses proposed to explain the phenomenon is that T6P can restrict sugar flux by inhibiting hexokinase activity (Paul *et al*., 2008).

In the nematode *Caenorhabditis elegans*, mutation of a TPP gene, *gob-1* (gut obstruct), is lethal. The mutation is thought to be lethal owing to the toxic buildup of T6P, suggesting that controlled levels of T6P are required for metabolic regulation (Kormish & McGhee, 2005).

In plants, there is a strong correlation between T6P and sucrose content. In addition to regulating the utilization of sucrose the amounts of T6P also respond strongly to this sugar. The amount of T6P is inversely related to the amounts of hexose phosphates and UDP-glucose suggesting that T6P may regulate the amounts of these intermediates (Paul *et al*., 2008). Sucrose would have in T6P a dynamic, specific and rapid signal. T6P would provide a further level of communication that could integrate information on sucrose supply with other factors, such as environmental stress (trehalose pathway genes are strongly regulated by stress as well as by sugar). Furthermore, as it is not part of a major metabolite flux toward an important end-product, T6P levels can fluctuate without compromising other functions, as would happen for central metabolites such as glucose-6P and UDP-glucose, from which T6P is synthesized. Potentially T6P could also signal the availability of these central intermediates (Paul, 2008).

Some authors propose a signaling role for mal1P similar to that of T6P. (Decker *et al.*, 1999)

3.1. Maltokinase

As formerly stated Drepper *et al*. identified a maltokinase (Mak) in 1996, in cell-free extract of *Actinoplanes sp*. The enzyme was isolated from crude extracts of *Actinoplanes missouriensis* and characterized only in 2003 by Niehues *et al*.

The enzyme maltokinase can catalyze the ATP-dependent phosphorylation of maltose to maltose-1-phosphate (mal1P). This reaction product was identified in 1967, by Narumi and Tsumita, in extract of *Mycobacterium bovis* BCG.

The gene coding for Mak, Rv0127 in *M. tuberculosis* and MMar_0326 in *M. marinum*, is identified in several genomic databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) or Institut Pasteur (http://genolist.pasteur.fr/TubercuList/).

The *mak* gene is located adjoining the *treS* gene, the gene coding for the enzyme catalyzing the interconversion of maltose to trehalose, which suggests the inclusion of both genes in the same transcriptional unit and the involvement of the two enzymes in the same metabolic pathway. Several other organisms present the same genetic conFiguration and in others, like *M. smegmatis* (MSMEG_6514), the genes are even fused together into one bifunctional *treS/mak* gene, which reinforces the previous consideration (Mendes *et al*., 2010).

The *mak* gene was considered essential for *M. tuberculosis* growth (Sassetti *et al.*, 2003), implying a capital function in bacterial metabolism. In *A. missouriensis* Mak seems to be expressed constitutively, independently of the growth phase and of the

sugars present in the growth medium (Niehues *et al*., 2003). This points to a possible function in anabolism, or to a role of mal1P as an effector molecule.

Niehues *et al*. (2003) characterized the Mak as a monomeric enzyme, with an estimated molecular mass of 57 kDa. The enzyme followed Michaelis-Menten kinetics, with a Km of 2.6 mM for maltose and 0.54 mM for ATP. Apparently the enzyme had a higher affinity for the phosphoryl-group donor than for the sugar substrate. Maltokinase exhibited a high specificity for maltose, relatively to similar disaccharides. The specificity for ATP as the phosphoryl-group donor was high (CTP, GTP, TTP, and UTP, showed little or none significant activity). The pH optimum was between 7 and 9.

The authors observed that the Mak activity was independent from the physiological state (exponential/stationary phase) of the bacteria or the maltose content of the broth. Even in a minimal medium with glucose, L-arabinose or D-xylose as a carbon source, Mak activity was present.

Jarling *et al.* (2004), following Niehues *et al*. (2003) work, isolated Mak1 from *Actinoplanes missouriensis* and Pep2 (Mak) from *Streptomyces coelicolor*, located on a gene cluster similar to that of Mak1 in *A. missouriensis*. The authors partially characterized the enzymes, to confirm their identity, and concluded that their proprieties were similar to the enzyme previously characterized by Niehues *et al*. (2003). Although both proteins appeared to be distantly related to aminoglycoside phosphotransferases (APHs) neither Mak1 nor Pep2 did phosphorylate any of the tested aminoglycosides. Therefore the authors concluded that it is physiologically unlikely that Mak1 or Pep2 could act as enzymes conferring aminoglycoside resistance to their hosts.

Mendes *et al.* (2010) characterized, for the first time, a mycobacterial Mak, in this case the enzyme from *Mycobacterium bovis* BCG, an enzyme with 99-100% amino acid identity to the Mak from *M. tuberculosis* strains. The protein was monomeric in solution, with a molecular mass of *c.* 50.7 ±4.2 kDa. The enzyme was dependent on maltose (Km 2.52±0.40 mM) and ATP (0.74±0.12 mM), although GTP and UTP could be used to produce mal1P and residual activity could also be detected with maltotriose, maltoetraose, maltopentaose and maltoheptaose as phosphate acceptors (maltose contamination could not be excluded).. Divalent cations were required for activity and Mg^{2+} was the best activator. The enzyme was active between 20 and 65°C, with maximal activity at about 60°C and at 37°C its pH range was between pH 6 and 11. Similarly to the enzymes from *A. missouriensis* and *S. coelicolor*, only maltose served as the acceptor substrate but, unlike those organisms' proteins the M. bovis BCG maltokinase was able to use ATP, GTP and UTP with comparable efficiency. This substrate flexibility may reflect an absolute requirement for mal1P, corroborating the proposed essentiality of the *mak* gene in *M. tuberculosis*.

Objectives

Maltokinases (Mak) are present in nearly all mycobacterial genomes available (Mendes *et al*., 2010). Moreover, the corresponding gene in *Mycobacterium tuberculosis* (Rv0127) has been considered essential for the growth of this organism (Sassetti *et al.*, 2003). However, only recently has a mycobacterial Mak been successfully expressed to allow functional characterization (Mendes *et al*., 2010), nonetheless the physiological role of this enzyme remains to be fully elucidated. Therefore, in an effort towards unveiling the role of Mak in mycobacterial physiology we decided to analyze the transcriptional responses of the corresponding gene in *M. smegmatis*, a suitable model mycobacterium, under different nutritional conditions. We also decided to explore additional functions of the enzyme *in vitro*, namely by testing possible alternative substrates (phosphoryl acceptors) as well as the possibility of the involvement of this enzyme in the inactivation of aminoglycoside antibiotics. Concurrently, to confirm the proposed essentiality of Mak in *M. tuberculosis*, we selected the closely related model organism *M. marinum* as the tool for the construction of a *mak* conditional knock-out mutant, and thus, evaluate the effects of silencing the gene on the mutant's phenotype. This multifactorial approach provides the grounds to further our knowledge not only of this enzyme's physiological role but also of its integration in the general mycobacterial metabolism.

Chapter II Methods

1. Mak Purification

1.1. Cloning

The maltokinase (*mak*) gene from *M. bovis* BCG was previously amplified by PCR, cloned into the expression vector pET30a (Novagen) and expressed in *E. coli* by V. Mendes (PhD dissertation) and described in the detail in the following publication (Mendes V, Maranha A, Lamosa P, da Costa MS, Empadinhas N., (2010) BMC Biochem 11:21).

1.2. Strain, Culture Conditions and Overexpression

E. coli BL-21 strain containing the pET30a plasmid with the maltokinase gene was grown in a 5L fermentor, in LB-Lennox medium with kanamycin (30 μg/mL), with continuous aeration and stirred at 180 r.p.m. at 37°C, pH 7.0. The bacteria grew to mid-exponential phase (OD_{610}=0.8). IPTG was added to a final concentration of 0.5 mM, to induce gene expression, and temperature was reduced to 20°C. The cells were harvested 18h after induction, by centrifugation (9000 × *g*, 10 min, 4°C).

1.3. Purification of the Recombinant Protein

The maltokinase purification protocol was performed based on Mendes *et al*., (2010). In an attempt to optimize the purification procedure, particularly to enhance the separation of the His-tagged Mak and simultaneously reduce the number of chromatographic steps, the pH of the sodium phosphate starter buffer containing 20 mM imidazole and 500 mM NaCl, required for the nickel affinity chromatography, was not adjusted to 7.4 as indicated by the column supplier (GE Healthcare), but maintained at pH 11.0.

E. coli cells carrying the His-tagged recombinant Mak from *M. bovis* BCG were suspended in 20 mM sodium phosphate buffer at pH 11.0 with 500 mM NaCl and 20 mM imidazole. A protease inhibitor cocktail (Roche), 10 μg/mL DNAse I and 5 mM $MgCl_2$ were added to the suspension. Cells were disrupted twice in a French-press cell followed by centrifugation (15000 × *g*, 4°C, 30 min) and the suspension was then filtered through 0.45 μm cellulose filter (WVR). The recombinant protein was purified in a prepacked Ni-Sepharose high-performance column (His-Prep FF 16/10) equilibrated with 20 mM sodium phosphate, pH 11.0, 500 mM NaCl, and 20 mM imidazole. Elution was carried out with 500 mM imidazole and the purity of the fractions was determined by SDS-PAGE. A TLC (Thin Layer Chromatography) was made to discriminate the purest active fractions. These were pooled, diluted ten times with 25 mM BTP at pH 7.4 and loaded into a Q-Sepharose fast-flow column (Hi-Load FF 16/10), equilibrated with 25 mM BTP at pH 7.4 with 50 mM NaCl, and eluted by a linear gradient of NaCl (0 to 500 mM). A TLC was once again prepared to locate the purest active fractions. Concurrently a SDS-PAGE was preformed and the purest active fractions were pooled, concentrated by ultracentrifugation in 30 kDa cutoff centricons (Amicon), equilibrated with 25 mM BTP at pH 7.4 with 200 mM NaCl, and loaded into a Superdex 200 fast-flow column equilibrated with the same buffer. After SDS-PAGE analysis the active purest fractions were concentrated and equilibrated with 50 mM BTP at pH 7.4 with 200 mM NaCl.

1.4. Substrate Specificity

Maltokinase activity had already been tested for maltose, maltotriose, maltotetraose, maltopentaose, maltoheptaose and trehalose by V. Mendes incorporated in his PhD thesis work. However further substrate specificity determination was assessed for the

disaccharides cellobiose, gentiobiose, leucrose, melibiose, palatinose, sucrose, turanose and β,β trehalose and for the trisaccharide raffinose. Since the Mak protein had high sequence identity (>40%) with putative aminoglycoside phosphotransferases, the aminoglycoside antibiotics gentamicin and kanamycin, were also tested as possible phosphate acceptors (all from Sigma-Aldrich). Maltose was utilized as a positive control in both tests. The reaction mixtures (50 µL) containing 0.5 µg of pure recombinant Mak, 3 mM of each sugar substrate or 5mM of the aminoglycoside antibiotics, 10 mM $MgCl_2$ and 50 mM BTP at pH 8.0, were incubated at 37°C for 10 min. When required negative controls were included where pure recombinant Mak was replaced by water. The reaction products were visualized by TLC using acetic acid/ethyl acetate/water/ammonia 25% (6:6:2:1, v/v) and propanol/ethyl acetate/water/ammonia 25% (5:1:3:1, v/v) as independent solvent systems.

2. Transcriptional Responses

2.1. Strains and Culture Conditions

Mycobacterium marinum (DSMZ 44344 (Deutsche Sammlung von Mikroorganismen und Zellkulturen)) and *Mycobacterium smegmatis* strain mc^2155 (ATCC 700084 (American Type Culture Collection)) were grown in Middlebrook 7H9 Broth complemented with 0.2% of Tween 80 (Sigma), at pH6.6. Five different nutritional conditions were employed. A 2.5 g/L D-glucose (Molekula), D-trehalose (Sigma), D-maltose (Sigma), glycerol (Prolabo) or L-glutamate (Sigma) supplement was added to the medium after being filter sterilized through a 0.2 µm cellulose acetate filter (VWR). Glycerol was added and autoclaved directly with the medium.

Cultures were inoculated to an initial optical density OD_{610}=0.05. For preparation of cell-free extracts the cells were grown in 1 L metal capped Erlenmeyers containing 250 mL of culture medium, for RNA extraction purposes the cultures were grown in 300 mL metal capped Erlenmeyers containing 100 mL of medium, in a water bath shaker at 160 r.p.m. *M. smegmatis* cultures were grown at 37ºC, while *M. marinum* cultures were grown at 30ºC. Growth curves were made for all the nutritional conditions tested so that cells could be harvested in the same growth phase (late exponential and stationary phase).

2.2. Activity in Cell-Free Extracts

M. smegmatis cells grown in different nutritional conditions were harvested 17h after inoculation, by centrifugation (8000 × *g*, 10 min, 4°C). Cells were suspended in 20 mM BTP buffer at pH 7.0 and were disrupted in a French-press cell, followed by centrifugation (14000 × *g*, 4°C, 30 min). The cell-free extracts were then dialyzed in 20 mM BTP buffer at pH 7.0 to remove unwanted substances, namely metabolites that could be substrates for Mak.

Determination of protein concentration of the cell-free extracts was done according to the Bradford method (Bradford, 1976).

Cell free extract activity was determined spectrophotometrically (Jenway 6405 UV/Vis) by measuring the ADP produced upon maltose phosphorylation. The assays were performed at 37 ºC. 50 µL reaction mixtures containing 20 µg of cell-free extract, 50 mM BTP pH 8.0, 10 mM $MgCl_2$, 2 mM maltose and 2 mM ATP were stopped at different times (0, 3, 6, 9 min) by cooling on ice . The protein was inactivated by the addition of 5 µL of 1N HCl and neutralized with 5 µL of 1N NaOH. The amount of

ADP released was determined by measuring the decrease in absorption at 340 nm, after incubation of the sample with 3 U of pyruvate kinase and lactate dehydrogenase, 0.3 mM NADH and 2.0 mM phosphoenolpyruvate (all from Sigma-Aldrich), 2.5mM of $MgCl_2$, 2.5mM of KCl and 50mM Tris HCl pH 7.4 to 1 ml mixture (total volume) for 10 min at 30°C.

2.3. Total RNA Isolation

All plasticware and glassware were treated before use to ensure that they were RNase-free. Chloroform-resistant plasticware (eppendorfs) were washed with chloroform to inactivate RNases, rinsed with RNase-free water and finally with ethanol. Glassware was washed with 5% HCl and rinsed with RNase-free water. Afterwards all material was autoclavated. Electrophoresis tanks were cleaned with 0.5% SDS, thoroughly rinsed with RNase-free water, followed by ethanol and allowed to dry. Aquous solutions were treated with 0.1% diethyl pyrocarbonate (DEPC), incubated overnight at 37°C and autoclaved for 15 min to remove any trace of DEPC. Water was filtered through a 0.22 µm cellulose filter (VWR) prior to DEPC addition. Glass beads (0.1 mm, BioSpec Products) were washed with 5% HCl, rinsed with RNase-free water and baked in an oven at 160°C for 2 hours.

For cleaning work surfaces RNase Zap (Ambion) was used. Pipettets were cleaned with ethanol and filtered tips (DIAMOND® Filter Tips Gilson) were utilized to prevent aerosol-borne contamination.

Total RNA isolation was performed in duplicates, during exponential and stationary phase. The optimal amount of starting material was determined in order to attain approximately the same cell concentration throughout the samples. After testing several

culture volumes, the most favorable was settled to be a volume of 6 mL of culture at OD_{610}=0.8. Total RNA was isolated from *M. smegmatis* cells grown for fourteen hours. DO_{610} was measured and the amount of starting material was calculated. Subsequent total RNA purification was carried out with RNeasy Protect Bacteria Mini Kit (Qiagen) according to manufacturer's indications. Mechanical disruption was employed to prepare the bacterial cells lysates, a 2 mL Safe-Lock Eppendorf containing 1 mL of acid-washed glass beads and the sample was attached to a vortex for 2 minutes. After on column DNAse treatment the samples were tested by conventional PCR (95ºC, 3min followed by 45x (95ºC, 10 s; 55ºC, 10 s; 72ºC, 15 s) with additional 72 ºC, 15s) with RpoB primers, to rule out DNA contamination. RNA quality following isolation was checked by formaldehyde agarose gel electrophoresis (1.2%). Nucleic acid concentration and sample purity were measured using a NanoDrop 1000 (Thermo Scientific).

When DNA contamination was detected after the RNA extraction protocol, total RNA was further digested with RNase free DNase I (Ambion), according to manufacturers' instructions. The nucleic acids solution was diluted to 10 μg nucleic acid/50 μL. The DNase was heat-inactivated at 65ºC for 10 min and beforehand EDTA had been added to the mixture at a final concentration of 5 mM.

Successful elimination of DNA contamination was confirmed by conventional PCR, with RpoB primers, (3 min, 95ºC and 45x (10 s 95ºC, 10 s 55ºC, 15 s 72ºC) plus 15 s 72ºC) (EDTA concentration was taken into account). RNA integrity subsequent to the digestion was checked by formaldehyde agarose gel electrophoresis (1.2%). sample purity and nucleic acid concentration were determined using a NanoDrop 1000 (Thermo Scientific).

cDNA was generated using Superscript III reverse transcriptase (Invitrogen) with random hexamers primers, according to the manufacturer's instructions.

2.4. Experimental Design and Testing of Specific Amplification Conditions

Primers were designed using NCBI´s primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) with the default parameters except for melting temperature range (minimum 50ºC, maximum 60ºC, optimal 55ºC), PCR product size (minimum 150, maximum 200), organism (*M. smegmatis* or *M. marinum*) and database (genome (chromosomes form all organisms)).

The *rpoB* gene (the gene encoding the β subunit of RNA polymerase) was chosen as an endogenous control as the ideal internal standard should be expressed at a constant level at all stages of development and should be unaffected by the experimental treatment. In addition, an endogenous control should also be expressed at roughly the same level as the RNA under study (Bustin, 2000). However extensive evidence suggests that all genes are regulated under some conditions and there is no universal reference gene with a constant expression so any system relying on reference genes should be carefully validated (Kubista *et al.*, 2006).

Table I – Oligonucleotides sequences utilized for RT-PCR

Target	Primer name	Forward primer [5'-3']	Reverse primer [5'-3']	Amplicon size
rpoB	RpoB	TGTTCGTCACGGCTGAGTTC	CGGTGGACTTGTCGATGGTC	189
rpoB	RpoB2	GTTTCATTTGCCAAGCTCCG	GACATCGAGCCCGAGAAATC	197
rpoB	RpoB3	TTCATTTGCCAAGCTCCGTG	ACATCGAGCCCGAGAAATCC	194
mak	Mak	CCAGCGATTGGTGGATCAGG	CCGCCTTGTCCAGTTCGTAG	179
mak	Mak2	CCGATGAGCGTTACCAGGTG	TCGTCGATCAGCGACAACAG	154
mak	Mak3	TATCACAAGCTCGCGGACAC	GCGTACTCGAACGACCTCAG	197
treS	TreS	GAACCACACCTCCGATCAGC	CGAACGTCCAGTTCGACTCC	155

Optimal primer concentration, annealing temperature and primer specificity were ascertained foremost by conventional PCR and agarose gel electrophoreses (1%, 4v/cm). Amplification was obtained for *rpoB* with 800 nM, 80 nM and 8 nM, final primer concentrations, displaying stronger bands for higher primer concentration.

Real-time PCR assays were performed to reassess optimal primer concentration, annealing temperature, primer specificity and cycle number. Optimal annealing temperature was considered to be 55ºC for all primers. Final primer concentrations of 800 nM, 200 nM and 80 nM were utilized. Following quantification and melting curve analysis the ideal primer concentration was deemed to be 800 nM for all genes.

2.5. Real-Time PCR Assay

Real-time-PCR was performed in a LightCycler® Carousel-Based System (Roche Applied Science) and in a MiniOpticon Real-Time PCR Detection System (BioRad) utilizing the light cycler FastStart DNA Masterplus SYBR Green I (Roche). The RT-PCR was done according to manufacturer's instructions. The program employed consisted of 3min at 95ºC, followed by 45 cyles of 10 s at 95ºC, 10 s at 55ºC and 10 s (unless when specified) at 72ºC, additional 15 s at 72 ºC and melting curve analysis. The reaction mix contained 2 µL of each primer (10x concentrated), 4 µL of master mix (5x concentrated), 100 ng of cDNA (unless specified otherwise) and PCR-grade water to a final volume of 20 µL. Negative controls containing water instead of cDNA were included in each run to detect any DNA contamination.

A Validation Experiment to determine if the $\Delta\Delta C_T$ calculation was suitable was optimized with cDNA input spanning from 100 ng to 5 ng.

3. Tetracycline-Controlled Conditional Mutants

The construction design of the conditional mutant was carried out by V. Mendes (PhD dissertation).

3.1. Strain, Culture Conditions and Plasmids

E. coli DH5α, when necessary, were grown in LB-Lennox medium with gentamycin (15 mg/mL) (in order to select transformants with a plasmid carrying a marker for gentamycin resistance). Cells were grown in test tubes containing 5 mL of medium, in a water bath shaker at 160 r.p.m. at 37ºC. For solid medium 2% agar was added.

The pJQ200sk plasmid (cloning vector carrying a marker for gentamycin resistance) was used to construct the conditional mutant. pGEM®-T Easy (Promega) cloning vector or pUC57 (GeneScript) cloning vector were utilized to lodge the fragments necessary to the construction on the intermediary stages. The plasmid pTC-28S150x was the source of the kan cassette (KanR2) and the strong mycobacterial promoter Psmyc was amplified from the same promoter. PmyctetO, the TetR responsive element which confers tetracycline-inducible expression when tetR is co-expressed, was amplified from pTC0x1L plasmid (all from Addgene).

3.2. DNA Manipulations

The fragments KanR2, PmycTetO and Psmyc were amplified by PCR from the afore mentioned plasmids with a *Taq* DNA Polymerase (Invitrogen), according to manufacturer's instructions. PCR amplification was verified by agarose gel electrophoreses (1%, 4v/cm). The products were then recovered from the agarose gel with the JETQUICK Gel Extraction Spin Kit (Genomed) and cloned into DH5α in a

pGEM®-T Easy cloning vector (Promega), allowing blue/white screening. After plasmid extraction, from white clones, with GenElute™ Plasmid Miniprep Kit (Sigma Aldrich), restriction analyses was performed with PstI/BamHI and sequencing (AGOWA) was carried out with M13F and M13R primers. The remaining two fragments (MakF and TreSF) were synthesized by GenScript and supplied in the pUC57 cloning vector. The inserts were subsequently cloned into *E. coli* DH5α. After plasmid purification, as described above, and restriction with suitable enzymes (all from Takara) both to confirm the presence of the inserts and to be utilized in downstream procedures (*cf.* Table II), the fragments were recovered from the 1% agarose gel with the JETQUICK Gel Extraction Spin Kit (Genomed).

To allow insertion of DNA the pJQ200sk plasmid was linearized with XhoI/ApaI. The first fragment to be cloned into the plasmid (MakF) had been previously digested with the same restriction enzymes. The plasmid and fragment were linked together by a T4 DNA ligase (Promega) and cloned into *E. coli* DH5α host cells. Correct fragment insertion was confirmed by restriction analysis. For downstream applications the plasmid was digested with NdeI/PstI (Table II). The fragment was recovered from the agarose gel with the JETQUICK Gel Extraction Spin Kit (Genomed). The procedure was repeated for all the fragments, in sequential order, varying only in the restriction enzymes utilized.

Table II – restriction enzymes utilized for construction

Fragment	Restriction enzymes
pJQ200sk	XhoI/ApaI
MakF+pJQ200sk	PstI/NdeI
MakF+pJQ200sk+PmycTetO	BamHI/PstI
MakF+PmycTetO+pJQ200sk+ KanR2	BamHI/XbaI
MakF+PmycTetO+ KanR2+ pJQ200sk+ Psmyc	NotI/XbaI
MakF+PmycTetO+ KanR2+ Psmyc+pJQ200sk+ TreSF	-

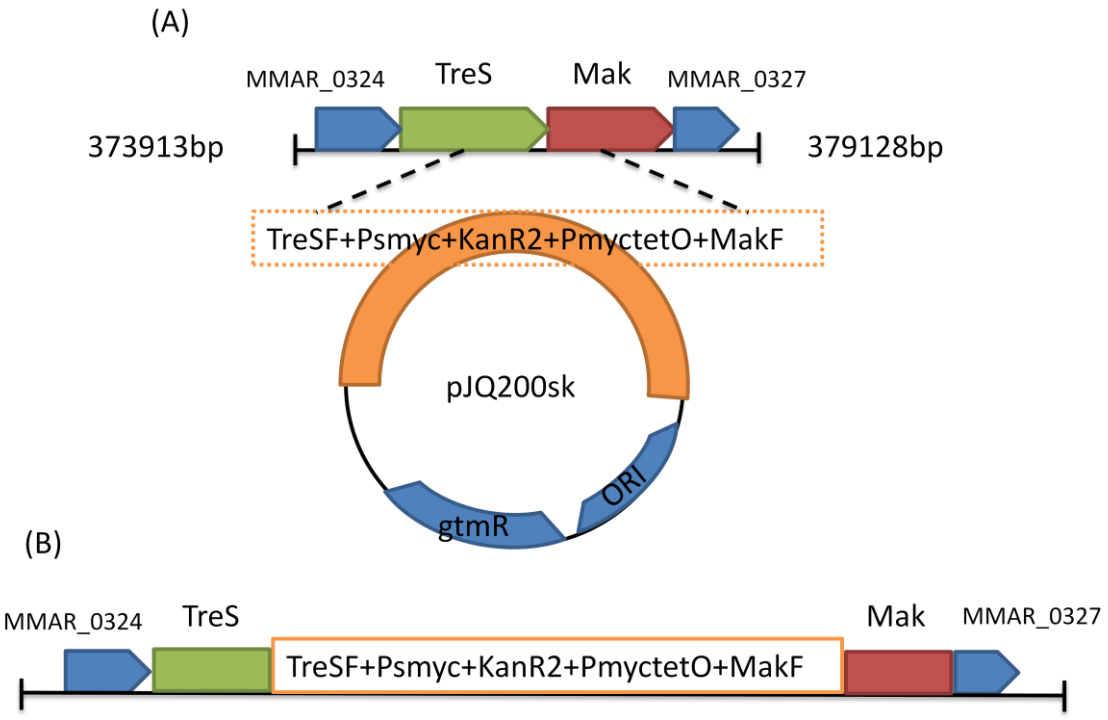


Figure 4 - Schematic overview of the expected knock-out mutant construction. Represented by the orange full hemicircle the vector´s MCS (multiple cloning site), the blue segments represent the ORI (Origin of Replication) and gtmR (gentamicin resistance gene) Integration of the TetR-responsive promoter PmyctetO, mycobacterial strong promoter Psmyc and kanamycin resistance cassette KanR2, via homologous recombination (black dotted lines). (A): *M. marinum*'s genome before homologous recombination (B): Expected result after homologous recombination

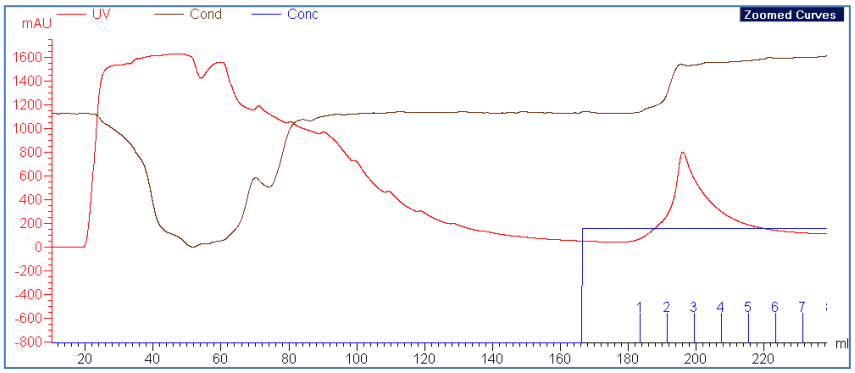
Chapter III Results and Discussion

1. Mak Purification

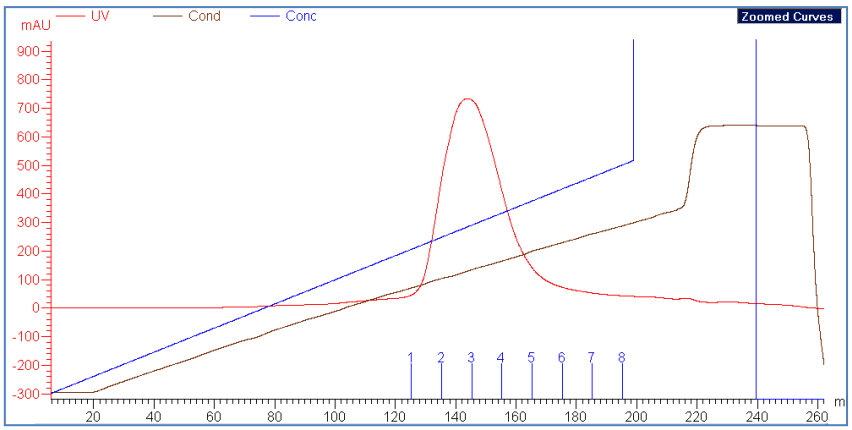
1.1. Purification of the Recombinant Protein

The most critical step in the study of any protein or enzyme is its purification. The development of techniques for protein purification has been pivotal for many advances in biotechnology. The basis of all protein purification is separation; cell-free extracts of biomaterial are complex mixtures of the protein of interest along with contaminating proteins and some small cellular debris. Separation is based on the protein's properties such as size, charge and the ability to bind, or be bound by, other molecules. The expression and purification of recombinant proteins facilitates characterization of proteins although often more than one purification step is necessary to reach the desired purity. The recombinant Mak protein was purified as described above in the Methods section. To achieve a better separation, and because the protein of interest had a broad pH range (pH 7 to 11, Mendes *et al*. (2010)), the sodium phosphate buffer was utilized at pH 11.0. The pH alteration allowed elution of purer fractions, narrowing down protein quantity for subsequent loads, which led to the purification of pure recombinant protein (Fig. 6) with the use of fewer columns. In fact the purity of the His-tagged recombinant Mak was high enough for biochemical characterization readily after the nickel-affinity column. The additional steps were performed to obtain a highly pure fraction for crystallography purposes and three-dimensional structure determination.

(A)



(B)



(C)

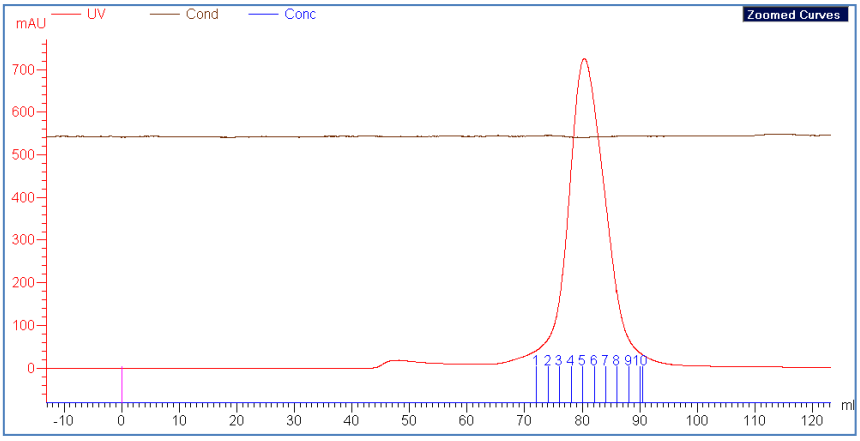


Figure 5 - FPLC chromatograms of the His-tagged Mak purification from cell-free extracts. (A): Column: Ni-Sepharose high-performance (His-Prep FF 16/10). Fractions 2, 3 and 4 were pooled diluted ten times with 25 mM BTP at pH 7.4 and loaded into the following column (B): Column: Q-Sepharose fast-flow column (Hi-Load FF 16/10). Fractions 2, 3, 4 and 5 were pooled (C): Column: Superdex 200 fastflow column. Fractions 3 to 8 were pooled concentrated and equilibrated with 50 mM BTP at pH 7.4 with 50 mM NaCl

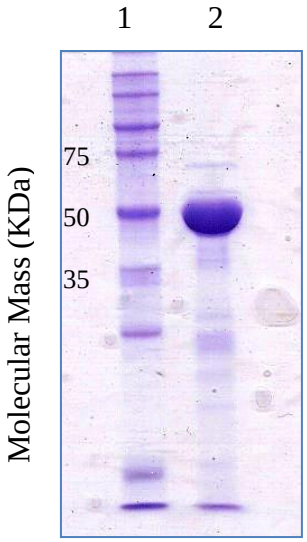


Figure 6 - SDS-PAGE (coomassie blue staining) of the fractions eluded from Superdex 200 fast-flow column following concentration by ultracentrifugation in 30 kDa cutoff centricons. Lane 1: molecular weight marker; lane 2: purified His-tagged Mak (50kD)

1.2. Substrate Specificity

In any organism phosphorylation does not happen spontaneously by mixing a substrate with ATP in aqueous solution. The coupling of an energy yielding reaction with an energy consuming one is catalyzed by enzymes. Enzymes also provide substrate specificity. Kinases are a ubiquitous group of enzymes central to many biochemical processes. The transfer of the terminal phosphoryl group to one substrate is a fundamental process in many aspects of metabolism, gene regulation and signal transduction.

Maltokinase activity had already been tested for maltose, maltotriose, maltotetraose, maltopentaose, maltoheptaose and trehalose (by V. Mendes PhD thesis work and (Mendes *et al*., 2010)).

In this work among the disaccharides and trissacharide substrates tested as possible phosphate acceptors no activity was detected, except for maltose, the positive control

(Fig. 7) which agrees with Niehues *et al*. (2003) and Jarlin *et al*. (2004) conclusions that the *Actinoplanes missouriensis' Streptomyces coelicolor*' Mak also exhibit a high specificity for maltose.

Aminoglycoside antibiotics are a large family of antibiotics that kill bacteria by binding to the bacterial ribosome and reducing fidelity of protein synthesis or inhibiting protein synthesis. The major mechanism of aminoglycoside resistance is enzymatic modification. Aminoglycoside phosphotransferases (APHs) perform cofactor-dependent drug modification in the bacterial cytoplasm; modified aminoglycosides bind poorly to the ribosome allowing bacteria to survive in the presence of the drug (Valulenko & Mobashery, 2003). Due to the sequence similarities of Mak with putative aminoglycoside phosphotransferases (>40%), the aminoglycoside antibiotics gentamicin and kanamycin were also tested as possible acceptors of phosphate but no activity was detected (Fig. 8). This is in accordance with the authors Jarling *et al*. (2004) findings for *Actinoplanes missouriensis* and *Streptomyces coelicolor* Mak proteins.

In *M. smegmatis* maltose appears to be highly specific as an acceptor of the phosphate group.

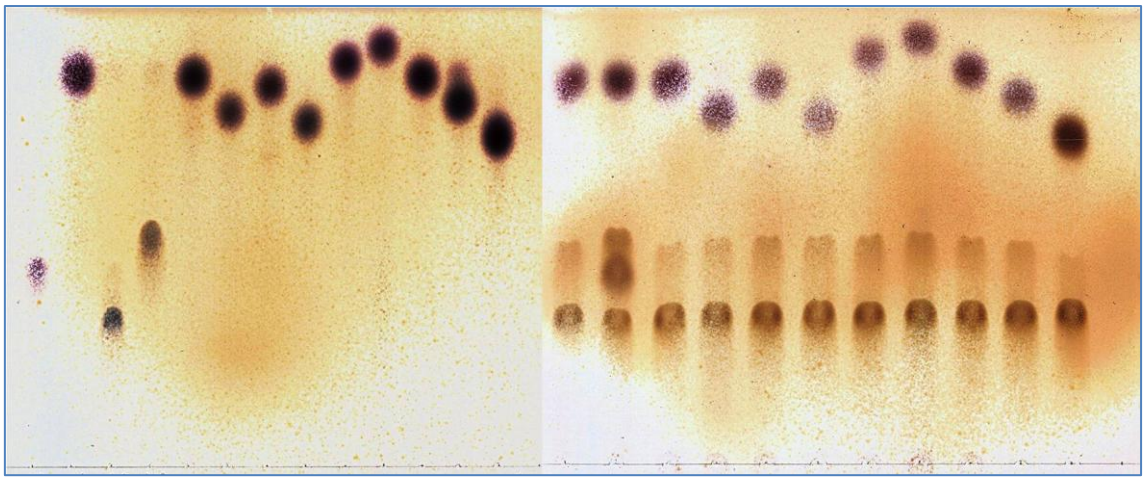


Figure 7 – Reaction products of the disaccharides and trisaccharide substrates tested as possible phosphate acceptors for Mak, visualized by TLC. Acetic acid/ethyl acetate/water/ammonia 25% (6:6:2:1, v/v) used as a solvent system. From left to right mal1P, maltose, ATP, ADP, cellobiose, gentiobiose, leucrose, melibiose, palatinose, sucrose, turanose, β,β trehalose and raffinose utilized as references, followed by a negative control (for the maltose reaction), the maltose reaction and the disaccharides and trisaccharide reaction, in sequential order (as previously described in the Methods)

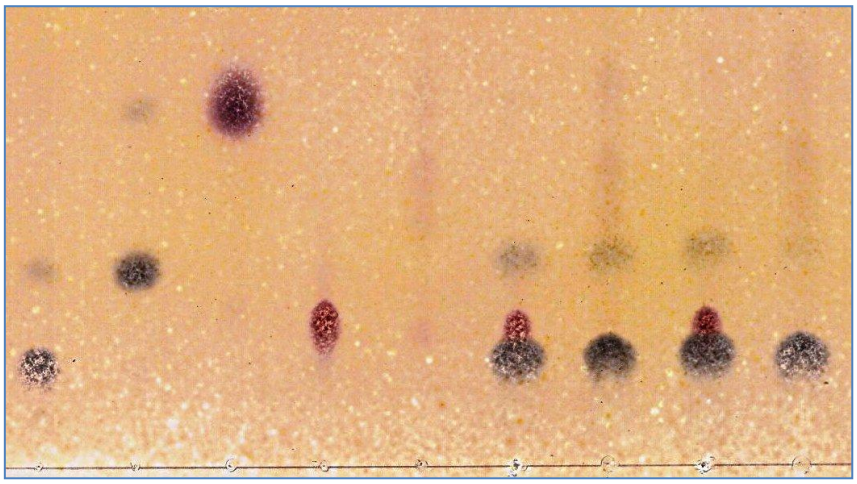


Figure 8 – Reaction products of the aminoglycoside antibiotics gentamicin, and kanamycin tested as possible phosphate acceptors visualized by TLC, utilizing propanol/ethyl acetate/water/ammonia 25% (5:1:3:1, v/v) as a solvent system. From left to right ATP, ADP, glucose, kanamycin and gentamicin, applied as references, followed by the kanamycin and gentamicin reactions and corresponding negative controls (as previously described in the Methods)

2. Transcriptional Responses

2.1. Influence of Carbon and Nitrogen Sources on Growth

Bacterial requirements for growth include sources of energy, organic carbon (e.g. sugars). Some carbon sources are utilized preferentially to others, favored sugars such as glucose, as long as they are present in sufficient amounts in the growth medium, repress the synthesis of the enzymes necessary for the transport and metabolism of less favorable carbon sources. This phenomenon became known as carbon catabolite repression (CCR). Deutscher *et al*. (2006) define CCR more broadly, as the inhibitory effect a certain carbon source has on gene expression and/or the activity of enzymes involved in the catabolism of other carbon sources.

Metabolic control at the enzymatic level is a function not only of the enzyme concentration, and thereby gene expression, but also of the interaction between enzymes and metabolites. Substrate availability (concentration) depends on active membrane transport systems (pumps, transporters) and the passive diffusion via substrate specific membrane proteins (ion channels, facilitators). Within a pathway there is a very tight control of fluxes such that the rates of synthesis and conversion of metabolites are kept in close balance over a very wide range of external conditions.

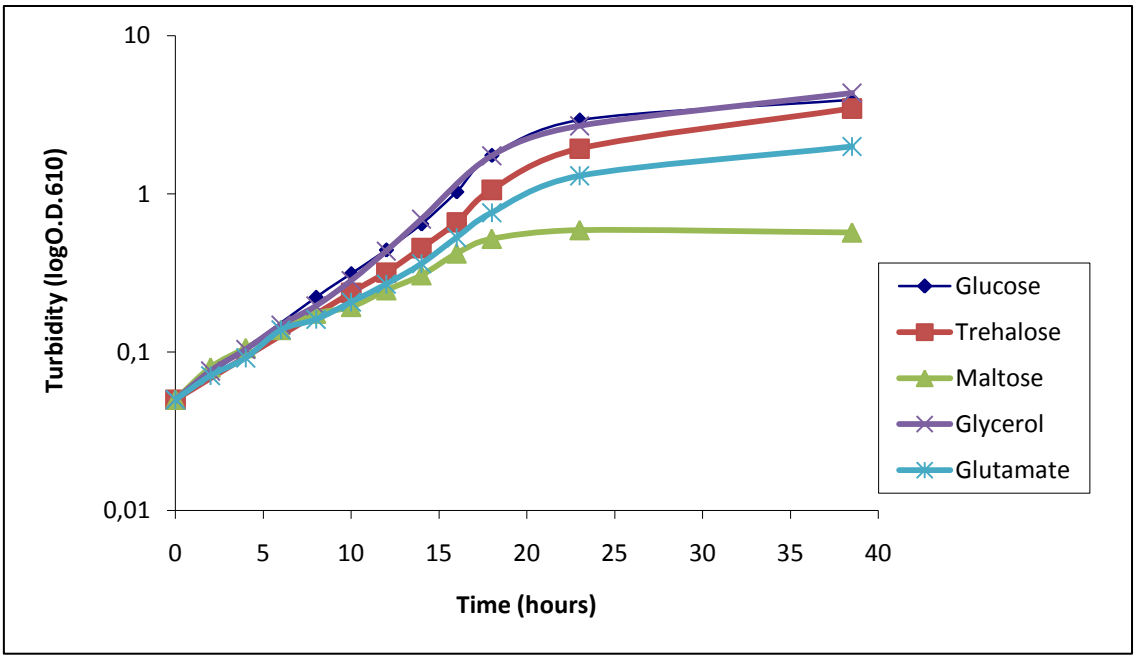
Owing to the complexity of metabolic networks, it is not obvious which reactions are essential and which are not, therefore, eliminating reactions overflow by growing organisms in sole carbon sources can be enlightening.

Despite the clear advantage of structuring biochemical reactions into pathways, it tends to leave the impression that these pathways are separate units that serve specific

purposes in the cell. This is by no means the situation and, the different pathways are often interconnected, resulting in a complex network of biochemical reactions.

As shown in Fig. 9A *Mycobacterium smegmatis* grows very well on glucose and glycerol, grows acceptably in trehalose and glutamate but grows poorly in maltose. Likewise *M. marinum* grows poorly on maltose but unlike *M. smegmatis* its favorite energy source is glutamate, followed by glucose, glycerol and trehalose (Fig. 9B). These nutritional preferences diverge from the ones observed by Wolf *et al*. (2003) and Tropis *et al.* (2005) in *Corynebacterium glutamicum.* The authors observed that the wild-type bacterium could not grow on trehalose but was perfectly capable of growing on maltose. However the authors Titgemeyer *et al*. (2007) detected poor growth on maltose as a sole carbon source in *M. smegmatis*, this being also true for *M. tuberculosis* (Edson, 1951). Pan *et al*. (2008) observe that, until now, it has not been clear where mycobacteria could obtain the maltose to transform into trehalose because *M. smegmatis* grows very poorly on maltose. This poses the conclusion that the metabolism involving these sugars must be particular to mycobacteria.

(A)



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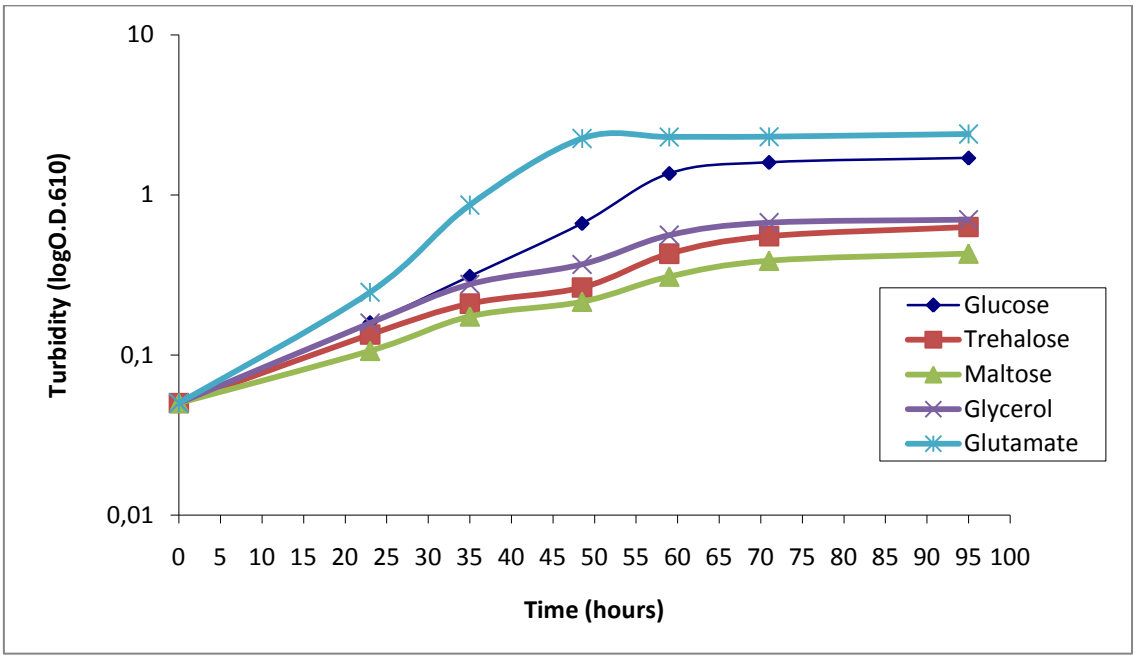


Figure 9 – Plot representing the growth of (A): *Mycobacterium smegmatis* (B): *Mycobacterium marinum* in 7H9 medium with glucose, thehalose, maltose, glycerol or glutamate as a carbon source

2.2. Activity in Cell-Free Extracts

The activity of maltokinase was measured as the rate of maltose-dependent formation of ADP from ATP. The ADP production was coupled to oxidation of NADH (β-nicotinamide adenine dinucleotide) by adding phosphoenolpyruvate (PEP) and purified pyruvate kinase and lactate dehydrogenase (*cf.* Fig. 10). The assay was performed in the presence of an excess of these reagents. Under these conditions, formation of ADP resulted in the oxidation of an equimolar concentration of NADH. A unit is defined as the amount of enzyme that catalyzes the removal of 1 µmole of substrate per min under standard assay conditions (Orston & Orston, 1969).

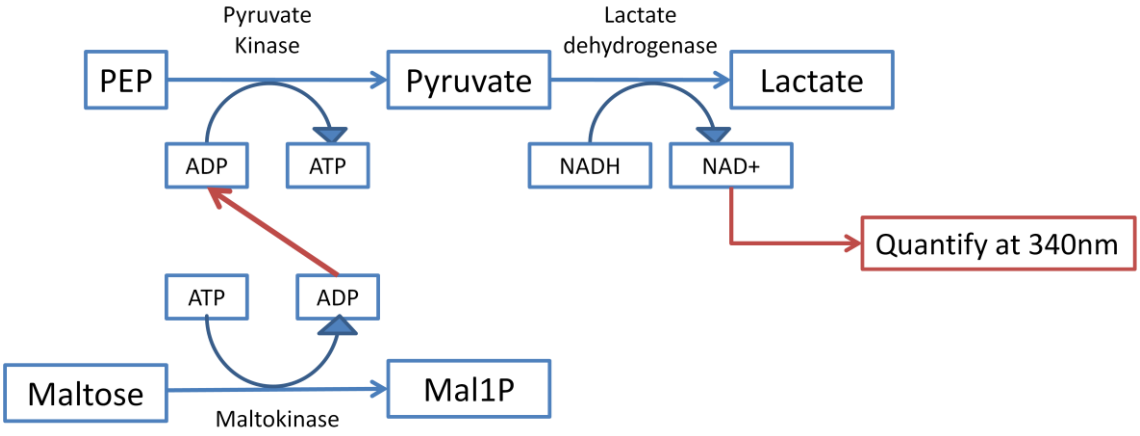


Figure 10 – Outline of the coupled reactions where ADP can be quantified by a loss of NADH

As shown in Fig. 11 there are no significant differences in the Mak activity among the carbon sources utilized, with the exception of maltose. The activity of the protein Mak in maltose was 1.52 times lower than in glutamate.

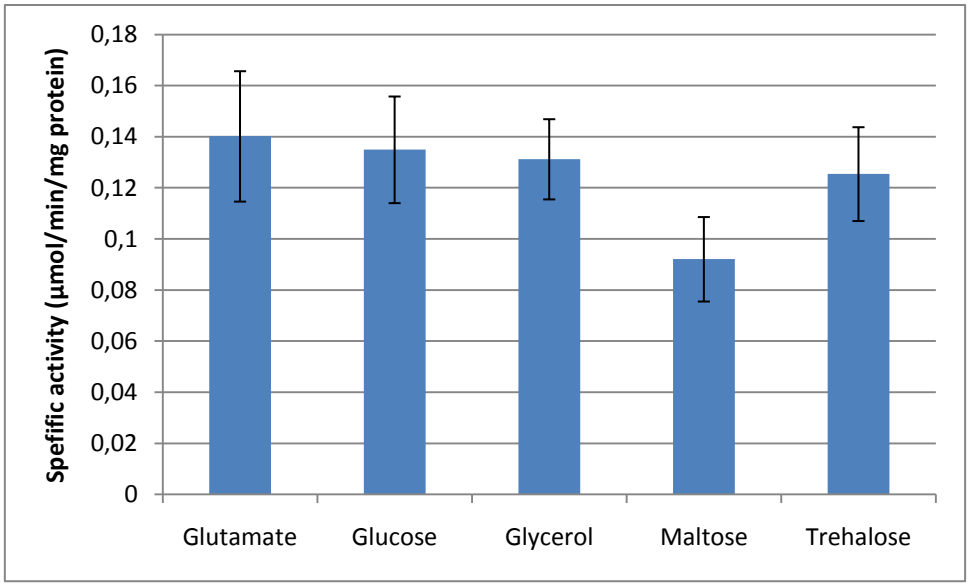


Figure 11- Specific activity of maltokinase in *Mycobacterium smegmatis* cell-free extracts grown in glutamate, glucose, glycerol, maltose and trehalose

The low enzyme activity on maltose coupled with the fact that *M. smegmatis* grows poorly on maltose might indicate the lack of uptake of this sugar, although M. *smegmatis* has several homologues for maltose transporters and this sugar was suggested to be transported by the permease ABC^{Sug}. The similarities of the ABC systems (ATP-binding cassette (ABC) transporter family) to known transporters outside the genus *Mycobacterium* is so low (<25 %) that substrates of these transporters cannot be predicted (Titgemeyer et al., 2007). Glycerol is used as the standard carbon source to grow *M. tuberculosis*; however, no specific uptake system is known, or apparent, by sequence similarity (Titgemeyer et al., 2007). Since *M. tuberculosis* grows with a generation time of 24 h and it has been shown that glycerol can directly diffuse through lipid membranes, it is conceivable that the rate of glycerol intake by passive diffusion may be sufficient for growth. Incoming glycerol would then be converted by glycerol kinase (GlpK) to glycerol-3-phosphate (G3P), which could then be converted by dehydrogenation to dihydroxyacetone phosphate (DHAP) by the enzyme glycerol-3-

phosphate dehydrogenase. DHAP can then be rearranged into glyceraldehyde 3-phosphate (GA3P) by triose phosphate isomerase (TIM), and feed into glycolysis (*cf.* Fig. 12).

Glutamate has a long history of being a preferred substrate of mycobacteria. In 1951 Edson reviews an increase in the measured the oxygen consumption in the presence of glutamate and reports this amino acid as one of the few nitrogenous substances that the bacteria is capable of oxidize. In 1963 Lyon *et al*. described some of the proprieties of the glutamate uptake system and concluded that a specific transport system for this amino acid should exist. Once glutamate has entered the cell via diffusion across the cytoplasmic membrane, or by protein-dependent transport, it is assimilated thanks to the glutamate dehydrogenase (GDH) that converts it to α-ketoglutarate, which can enter central metabolism via the Krebs cycle (*cf.* Fig. 12).

From the work of Woodruff et al. (2004) that disabled key-enzymes required for each of the three trehalose synthesis pathways in *M. smegmatis* resulting in a mutant unable to proliferate unless supplemented with trehalose, it can be concluded that an uptake system of a sort must exist. Titgemeyer et al. (2007) also propose the existence of an uptake system exist for this sugar after growing *M. smegmatis* in trehalose as the sole carbon source.

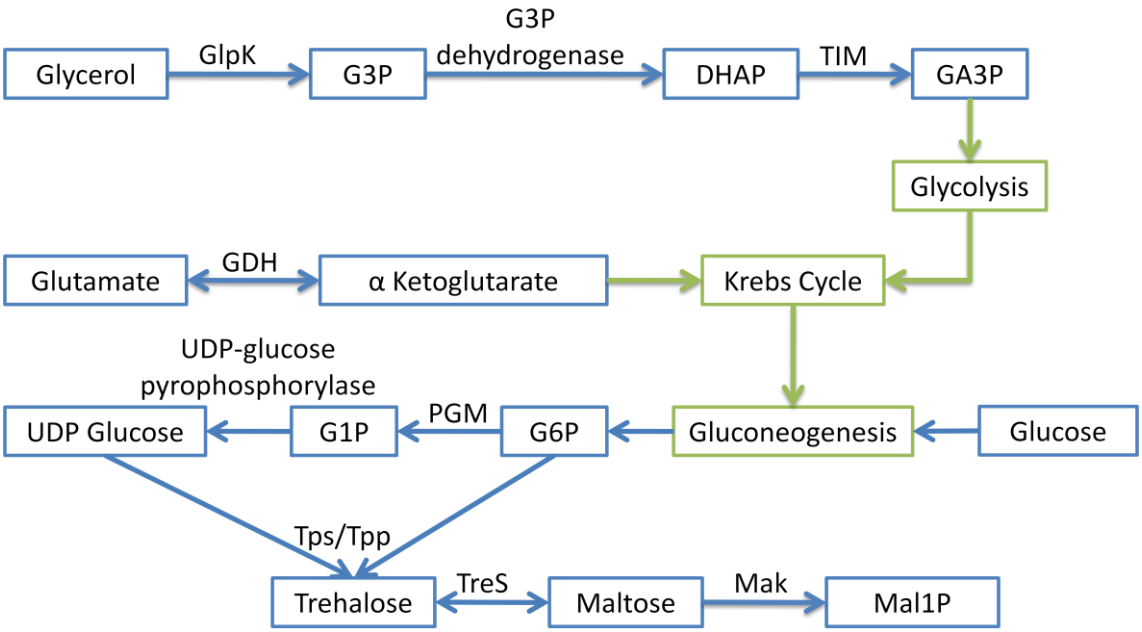


Figure 12– Integration of the metabolic pathways of the carbon and nitrogen sources utilized leading to maltose-1-phosphate production

2.3. RNA Isolation

Obtaining high quality, intact RNA is the first, and often the most critical, step in performing RT-PCR. Starting with low quality RNA may strongly compromise the results of downstream applications. Obtaining high quality RNA is determinant to the reproducibility and biological relevance of subsequent procedures.

To ensure reliable gene expression analysis RNA should ideally be stabilized *in vivo*. Bacterial mRNAs usually have a very short half-life, often only a few minutes and genes may be induced during handling and processing of bacterial cells, leading to higher expression of specific genes.

Many cellular decisions are reflected in altered patterns of gene expression. The ability to quantitate transcription levels of specific genes is central to any research into gene function. RT-PCR is the most sensitive and flexible quantification method and it can be used to compare the levels of mRNAs in different sample populations, to characterize patterns of mRNA expression, or to discriminate between closely related mRNAs. Nevertheless, its successful application depends on careful experimental design and validation remains essential for accurate quantitative measurements of transcription (Bustin, 2000; Bustin & Nolan, 2004).

The optimal amount of starting material for total RNA extraction was determined in order to attain approximately the same cell concentration throughout the samples. After testing several culture volumes, the most favorable was settled to be a volume of 6 mL of culture at OD_{610}=0.8.

As shown in Fig. 13 agarose gel electrophoresis following PCR amplification of total RNA extraction revealed that most samples were contaminated with genomic DNA. RNA quality was checked by denaturing gel electrophoresis (formaldehyde agarose) the visualization of sharp 23S and 16S rRNA bands (as opposed to smeared bands) is a good indication that the RNA is intact (Fig. 14).

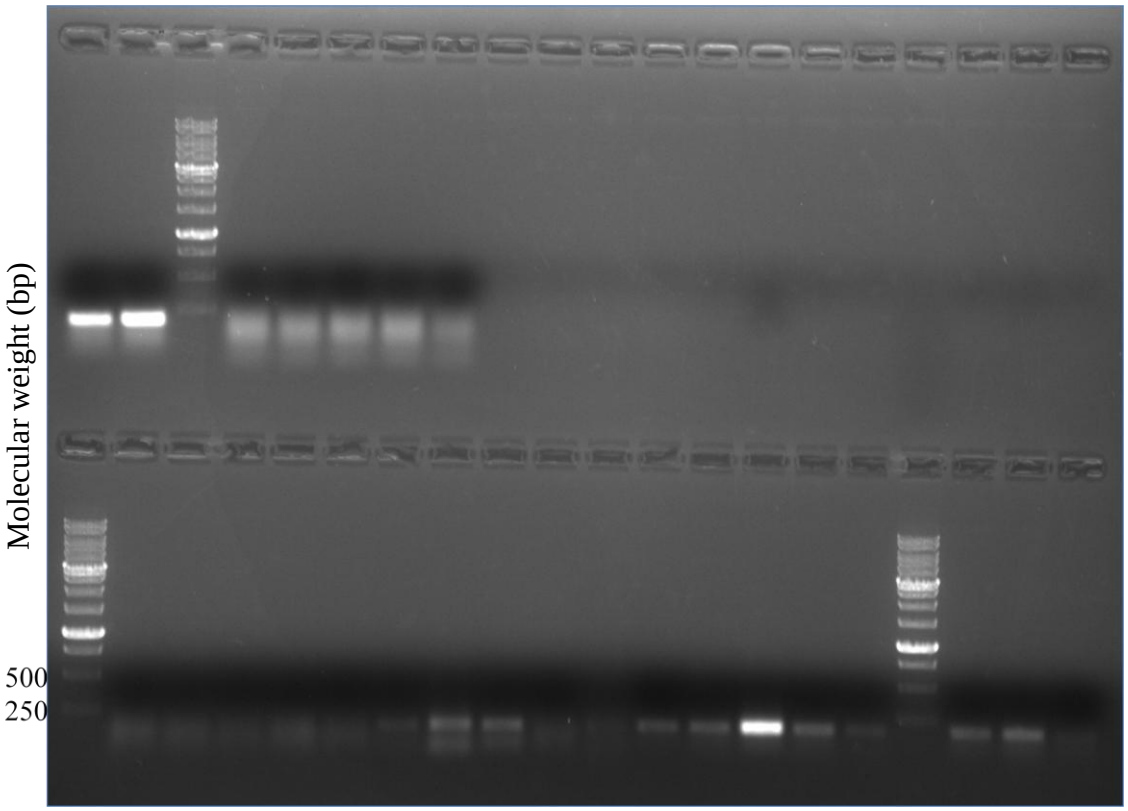


Figure 13 – PCR products examined via ethidium bromide-stained agarose gel electrophoresis (2%) following RNA extraction and DNase on column digestion (Bioron 1Kb DNA ladder). From the bottom left to the upper right. Samples extracted from *M. smegmatis* in exponential phase, grown in glucose, trehalose, maltose and glutamate (two replicates); samples extracted from *M. smegmatis* in stationary phase, grown in glucose, trehalose, maltose and glutamate (two replicates); samples extracted from *M. marinum* in exponential phase, grown in glucose, trehalose, maltose and glutamate

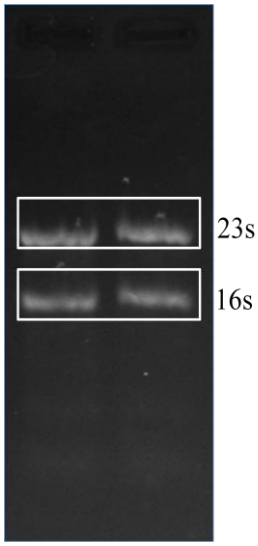


Figure 14 – Denaturing formaldehyde agarose gel (1.2%) following total RNA isolation examined via ethidium bromide-staining. Samples extracted from *M. smegmatis* in exponential phase, grown in glutamate

As Fig. 15 demonstrates after treatment with DNase I genomic DNA contamination was successfully eliminated. Controls were run with EDTA to account for possible Taq polymerase inhibition (Fig. 15 B). EDTA was added to the control PCR reaction in same final concentration as in the reaction containing RNA. RNA quality was once again checked by denaturing gel electrophoresis in a formaldehyde agarose gel (Fig. 17). The decrease in band intensity can be explained by some total RNA loss after digestion, as shown in Table III.

Although the addition of EDTA to the digestion reaction tainted the RNA samples (A260/230 ratios Table III) it was strictly necessary so that the RNA would not undergo chemical scission when heated, since magnesium and other metals catalyze non-specific cleavages in RNA. A protocol consisting of chloroform: isoamyl alchol (24:1) extraction, followed by precipitation with absolute ethanol overnight at -80ºC and washing with 70% ethanol RNase-free, in order to eliminate the DNase and the EDTA, was attempted several times. However this extraction protocol implied significant RNA losses whilst the heat-inactivating alone sufficed to decontaminate the RNA and the EDTA created no problems for any downstream procedures.

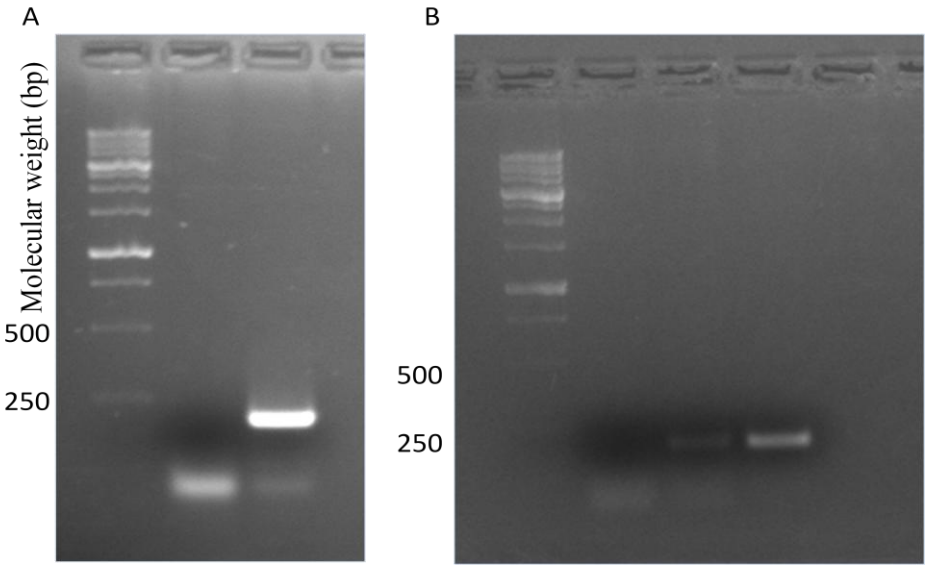


Figure 15 - PCR products examined via ethidium bromide-stained agarose gel electrophoresis (2%) following DNaseI digestion (Bionron 1Kb DNA ladder). (A): From left to right. Sample extracted from *M. smegmatis* in stationary phase, grown in glucose, positive control run with cDNA. (B): From left to right. Sample extracted from *M. smegmatis* in stationary phase, grown in glutamate, positive control run with cDNA with 0.2 mM EDTA and without EDTA

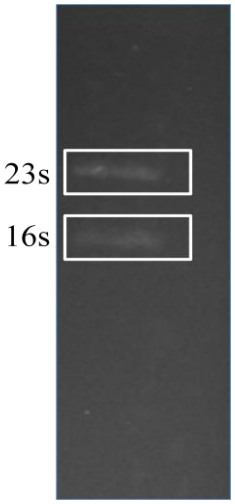


Figure 16 – Denaturing formaldehyde agarose gel (1.2%) following DNaseI digestion examined via ethidium bromide-staining. Sample extracted from *M. smegmatis* in stationary phase, grown in glutamate

Table III – RNA concentration and sample purity measured on a Nanodrop 1000, prior and after digestion with DNase I. Samples grown on Glutamate (Glut) and Glucose (Glu), RNA extracted in exponential or stationary phase (est)

Sample	**Prior to diggestion**			**After diggestion**		
	RNA concentration (ng/µL)	Sample purity (260/280 ratio)	Sample purity (260/230 ratio)	RNA concentration (ng/µL)	Sample purity (260/280 ratio)	Sample purity (260/230 ratio)
Glut	89.7	2.13	0.34	-	-	-
Glut2	363.6	1.90	1.86	-	-	-
Glu est 2	262.9	2.10	1.84	74.1	2.06	1.49
Glut est 2	400.6	1.92	2.01	151.4	1.88	1.31

2.4. Experimental Design and Testing of Specific Amplification Conditions

Optimal primer concentration, annealing temperature and primer specificity were ascertained foremost by conventional PCR and agarose gel electrophoreses (2%, 4v/cm). Amplification was obtained for *rpoB* with 800 nM, 80 nM and 8 nM, final primer concentrations, displaying stronger bands for higher primer concentration. The *mak* and *treS* amplicons were not visible after gel electrophoreses. Annealing temperatures ranging from 50 to 60ºC (50, 52, 55, 58 and 60ºC) were tested. No amplification was obtained for 50ºC. For 52 and 58ºC amplification held some unspecific products, while for 60ºC gel analysis showed a very weak band. Annealing temperature at 55ºC obtained optimal amplification. No conclusion could be drawn from conventional amplification and agarose gel electrophoreses (2%) in regard to the *mak* and *treS* amplicons for they were not visible after gel electrophoreses.

Real-time PCR assays were performed to reassess optimal primer concentration, annealing temperature, primer specificity and cycle number. Optimal annealing temperature was considered to be 55ºC for all primers. Final primer concentrations 800 nM, 200 nM and 80 nM were utilized (Fig. 17). Although primer concentration did not appear critical to RpoB amplification (Fig. 17 B) it had an extreme influence on TreS amplification (Fig. 17 D). Agarose gel analysis was congruent with melting curve analysis showing that primer concentration had a great impact on TreS amplification and primers concentration 200 nM and 80 nM were insufficient for optimal amplification. In regard to RpoB there was also a decrease in amplification although not so pronounced (Fig 18). Following quantification, melting curve analysis and agarose gel electrophoresis the ideal primer concentration was deemed to be 800 nM for all genes (Fig. 18).

For the *mak* and *rpoB* amplicons six primers were tested but only Mak3 and RpoB2 were target specific and did not originate primer dimers. Mak amplified a non specific product with *c*. 400 bp (Fig. 18 in blue) and Mak2 was prone to dimer formation. RpoB was liable to mispriming and both RpoB and RpoB3 originated primer dimers (although RpoB3 in inconsequential amounts) (Fig. 20-88ºC peak and Fig. 21 signaled in a black dotted line). RpoB negative control shows amplification product not only due to primer dimer formation but also to DNA contamination (Fig 19 blue line). However, the difference in C(t) (threshold cycle - cycle at which the fluorescence crosses the threshold (level of detection of fluorescent intensity above background)) (Table V) for the various amplicons in Fig. 20 can be explained due to primer dimer formation in the control reaction. Despite having no amplicon amplification on the negative controls (Fig. 20 TreS amplicon peak at 90.5ºC vs. negative control dimer peak at 80.5ºC or Mak3 amplicon peak at 92.5ºC vs. negative control dimer peak at 84ºC) primer dimer prevents the absence of amplification. Mispriming can also be problematic (Fig 20 RpoB peak 88ºC), once again preventing the occurrence of negative controls (for a better understanding of the amplification plots *cf.* Fig. 19 to 21 with Tables IV to VI).

Primer dimer formation can be challenging when trying to obtain a negative control because SYBR Green dye will bind to any double stranded DNA molecule, as such any primer dimers will cause higher background and may lead to amplification on the negative controls. The presence of primer dimers can be easily identified by the presence of additional peak or peaks in the melting curve analysis. The primer dimer peaks usually appear at low melting temperatures. Mispriming can also be a problem because it will leave less primer available for annealing to the target sequence and amplification of a non specific sequence will present erroneous C(t) values.

At this point annealing time was lowered from 15 seconds to 10 seconds in order to prevent the amplification of non specific products. 45 cycles, as recommended by the FastStart DNA Masterplus SYBR Green I (Roche) manufacturers', was considered ideal to cover low starting concentration of template DNA.

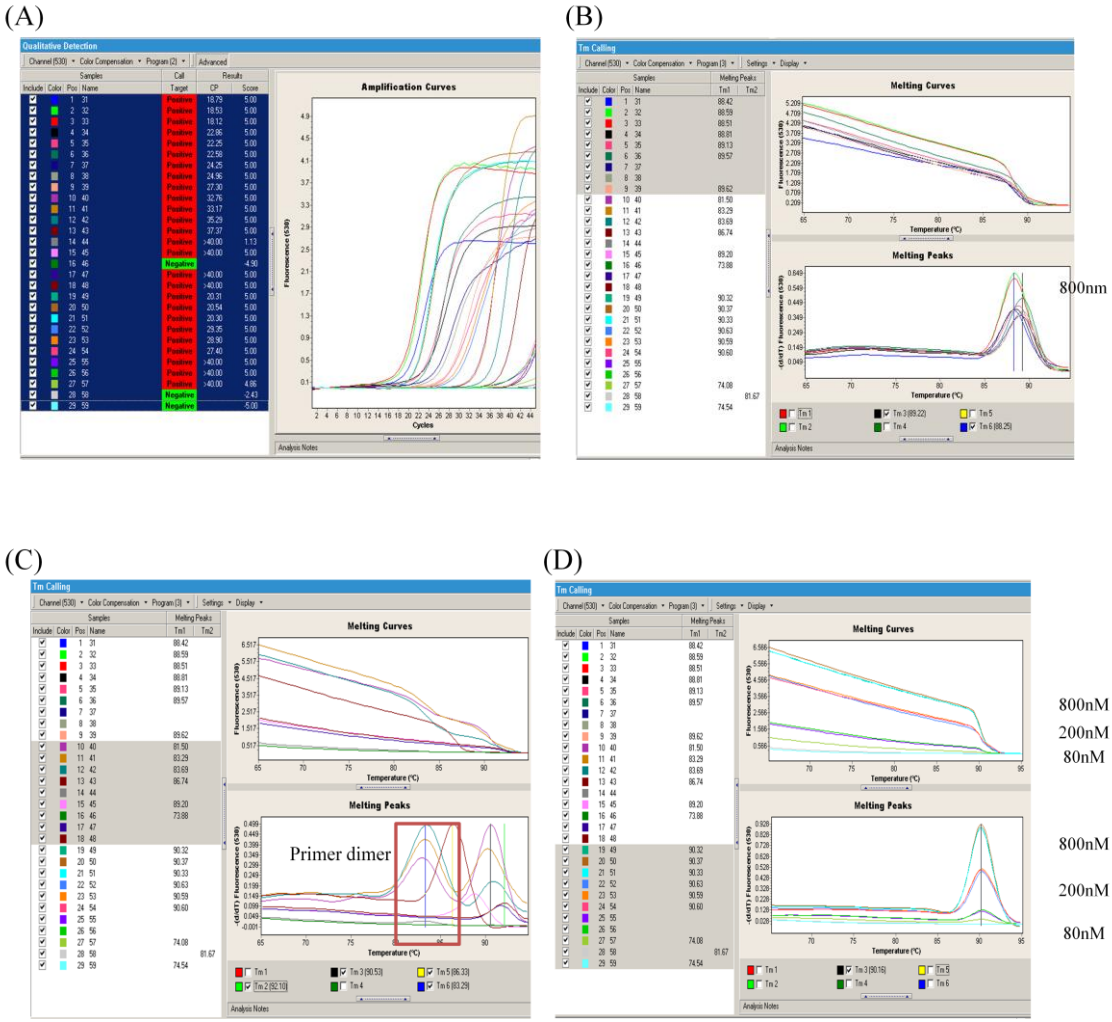


Figure 17 - Quantification analysis of RT-PCR products from a SYBR Green I assay. (A): Amplification curve for all samples (B): Melting curve analysis for RpoB primer concentration 800 nM, 200 nM and 80 nM (C): Melting curve analysis for Mak primer concentration 800 nM, 200 nM and 80 nM (D): Melting curve analysis for TreS primer concentration 800 nM, 200 nM and 80 nM

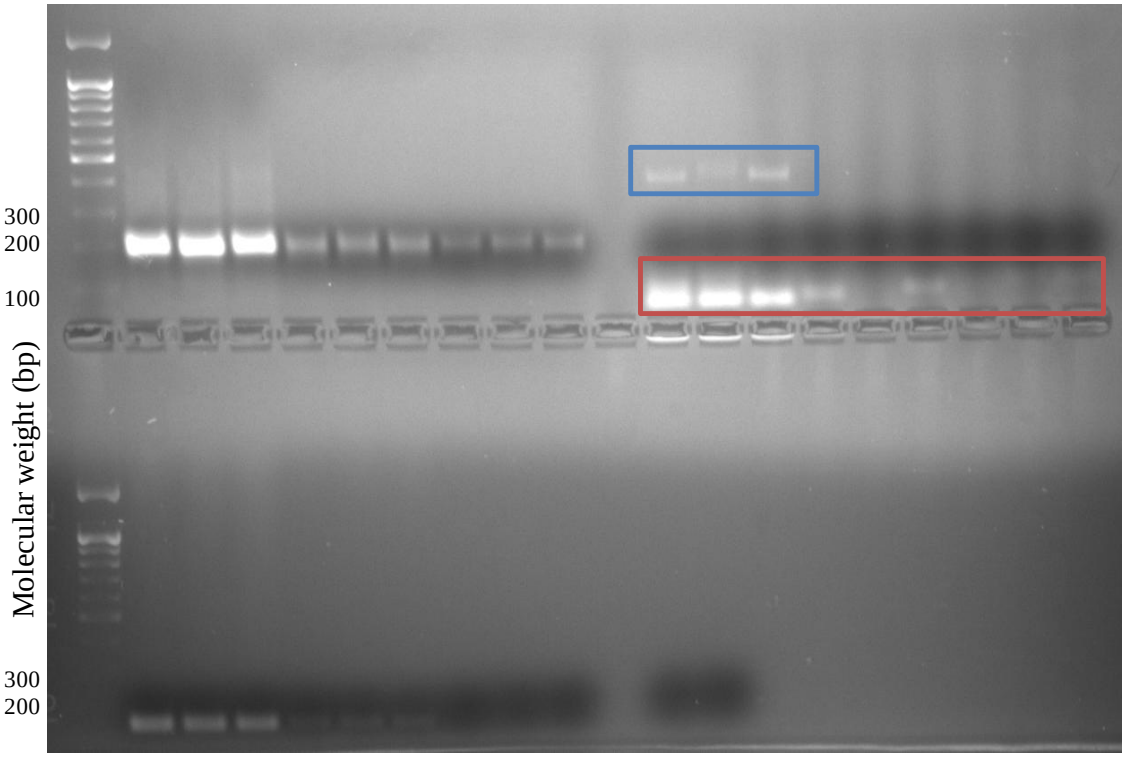
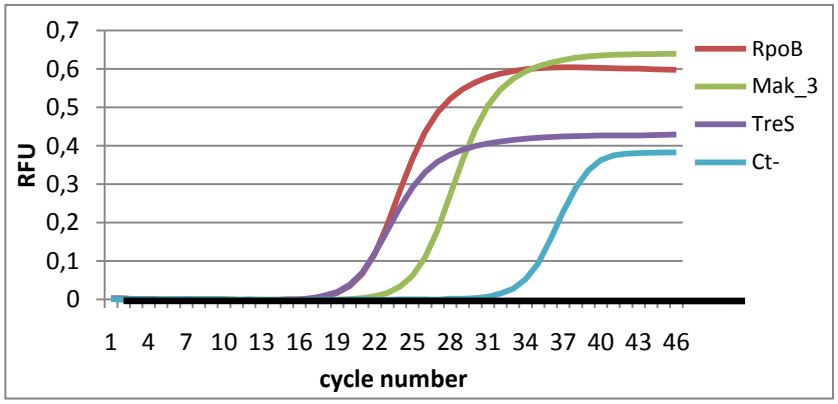


Figure 18 - RT-PCR products examined via ethidium bromide-stained agarose gel electrophoresis (2%) (Bioron 100 bp + 1.5 kb DNA Ladder). From the upper left to the bottom right (reactions in triplicates) RpoB primers final concentrations 800 nM, 200 nM and 80 nM, Mak primers, TreS primers, negative controls (as previously described in the Methods). Signaled in blue Mak non specific product with *c.* 400 bp. Signaled in red primer dimer formation by the Mak primer

(A)



(B)

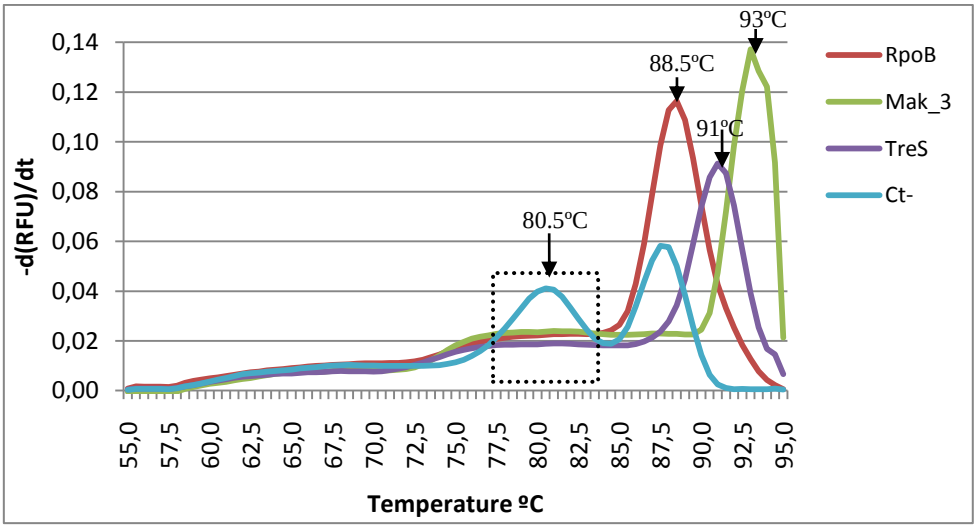
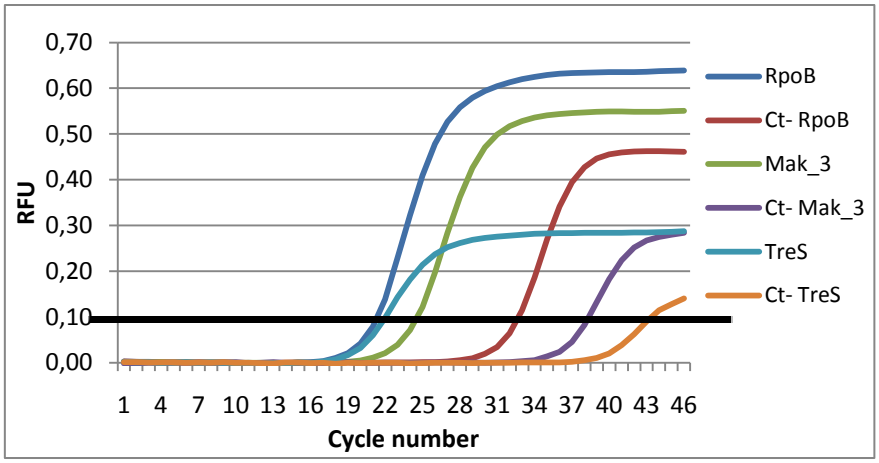


Figure 19 - Quantification analysis of RT-PCR products from a SYBR Green I assay. (A): Amplification Curve; relative florescence units plotted as a function of cycle number. Black line representing the threshold line (B): Melting analysis; the negative first derivative of the change in fluorescence plotted as a function of temperature

Table IV – Table showing the C(t) of RT-PCR products from a SYBR Green I assay

Sample	C(t)
RpoB	21.33
Mak3	25.46
TreS	21.27
Ct- RpoB	34.77

(A)



(B)

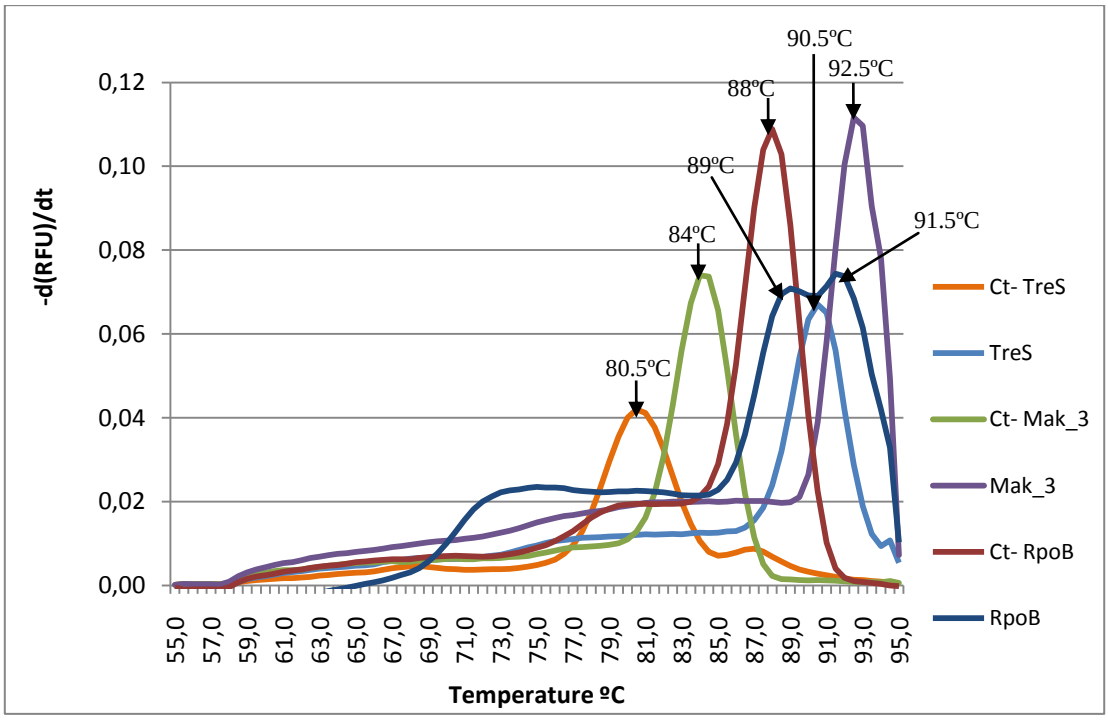
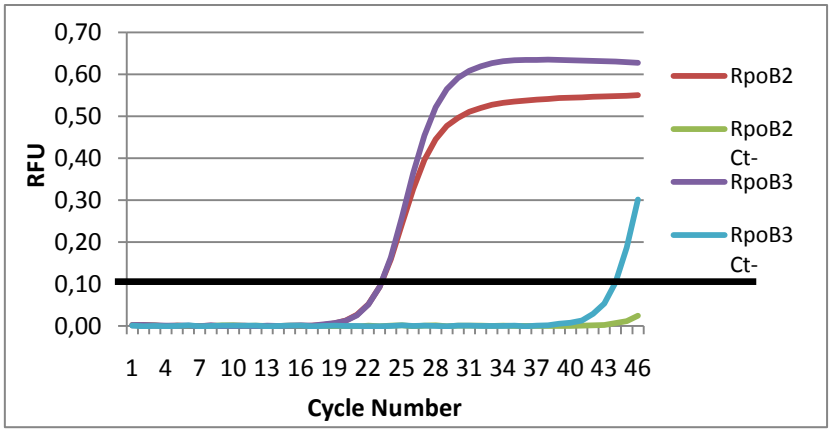


Figure 20 - Quantification analysis of RT-PCR products from a SYBR Green I assay. (A): Amplification Curve; relative florescence units plotted as a function of cycle number. Black line representing the threshold line (B): Melting analysis; the negative first derivative of the change in fluorescence plotted as a function of temperature

Table V – Table showing the C(t) of RT-PCR products from a SYBR Green I assay

Sample	C(t)
RpoB	20.93
Ct- RpoB	32.23
Mak	24.09
Ct- Mak	37.83
TreS	21.42
Ct- TreS	42.49

(A)



(B)

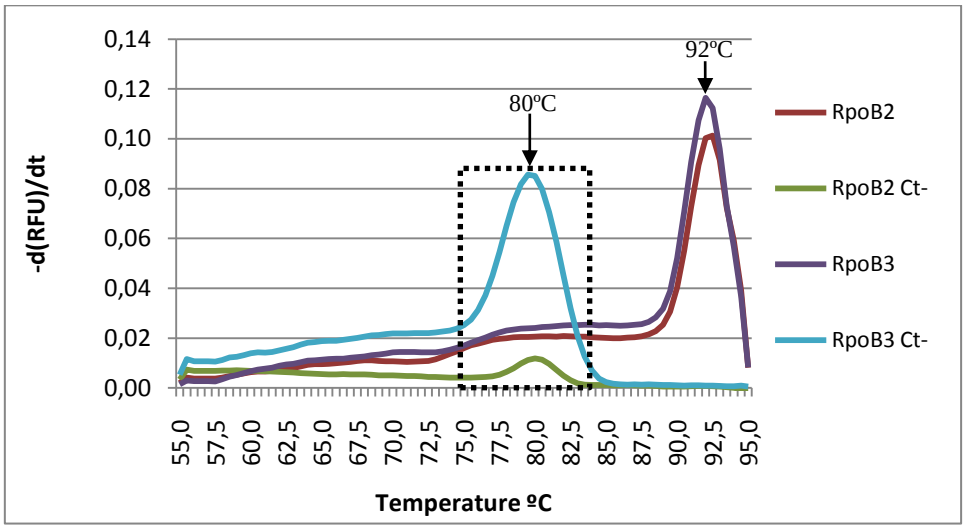


Figure 21 - Quantification analysis of RT-PCR products from a SYBR Green I assay. (A): Amplification Curve; relative florescence units plotted as a function of cycle number. Black line representing the threshold line (B): Melting analysis; the negative first derivative of the change in fluorescence plotted as a function of temperature

Table VI - showing the C(t) of RT-PCR products from a SYBR Green I assay

Sample	C(t)
RpoB2	23.07
RpoB2 Ct-	-
RpoB3	23.05
RpoB3 Ct-	43.87

2.5. Real-Time PCR Assay

When the validation of the $\Delta\Delta C_T$ experiment was started a series of technical problems were encountered. Contaminations, primer dimer formation and mispriming were common problems when trying to conduct an experiment. Several controls were inserted to try and locate the source of the contamination but no conclusion was drawn except that aerosol-borne contamination made reagent viability very short. To account for dimer formation more than one primer was used in an experiment but again no conclusion could be reached.

3. Tetracycline-Controlled Conditional Mutants

3.1. DNA Manipulations

Reverse genetic techniques are essential tools for studying genes vital for growth. The ability to express gene products in a temporally restricted manner has been an essential experimental strategy in determining gene function where mutant or knockout studies are not possible. A straightforward method for obtaining conditional mutants is to replace the native promoter of a target gene with a tightly regulated promoter whose activity can be controlled experimentally.

Tet repressor (TetR) proteins regulate the expression of a family of tetracycline (Tc) exporting proteins. The P_{tet} promoter expresses TetR. In the absence of Tc TetR binds the tet operator (tetO) in the tetA promoter and suppresses transcription of tetA, which encodes the Tc exporter. Once Tc enters the cell it binds TetR and induces a conformational change that results in dissociation of TetR from tetO and thus induces

expression of TetR controlled genes (*cf.* Fig. 22). Induction of TetR occurs prior to inhibition of the ribosome by tetracycline because affinity of TetR for these drugs is 10^3 to 10^5 fold higher than the affinity of the drugs for the ribosome. Tetracyclines can cross biological membranes by diffusion, enabling these inducers to penetrate most bacterial cells.

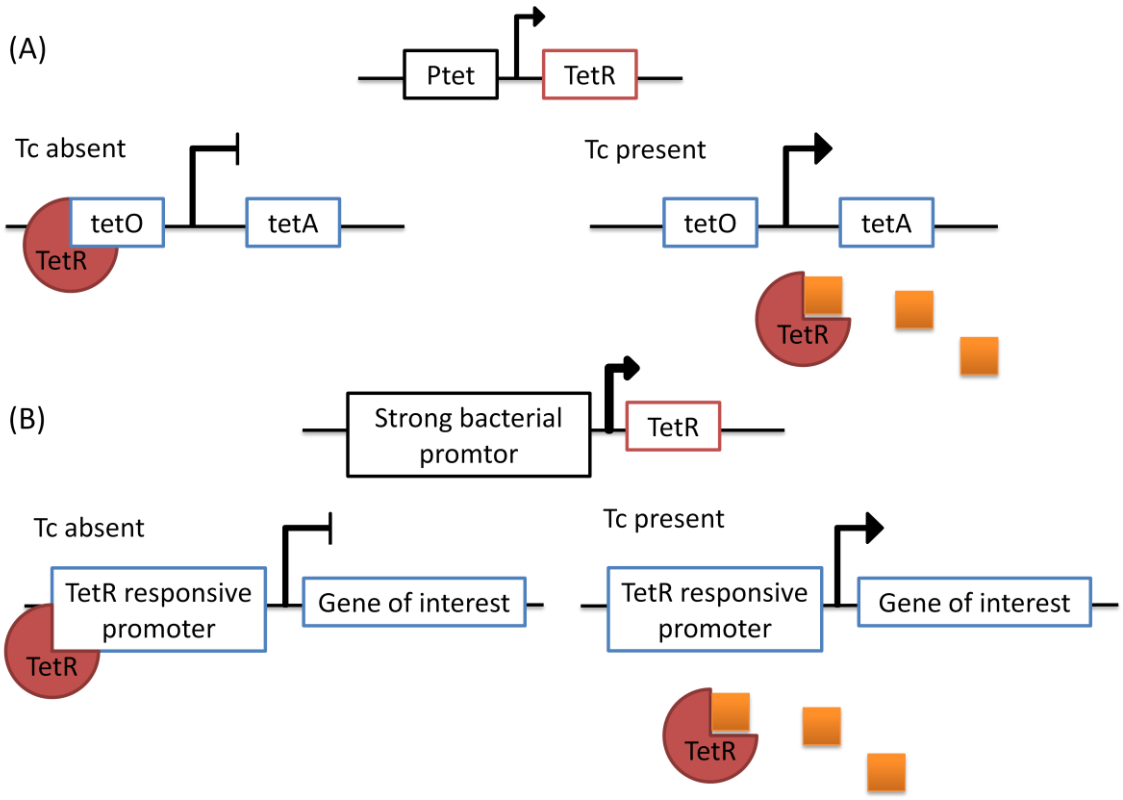


Figure 22 – Tetracycline regulated gene expression. (A): The Tet operon (B): The adaptation of the Tet operon to controlled transcriptional activation

Bacteria synthesize restriction endonucleases to protect themselves from attack by viruses. These enzymes recognize particular base sequences, recognition sites, and cleave that DNA at defined positions. Each species of bacterium protects its own DNA by methylation at the recognition site, so that only invading viral DNA is cleaved and

degraded. Because of the sequence specificity, the positions of cuts within a DNA molecule can be predicted, assuming that the DNA sequence is known, enabling defined segments to be excised from a larger molecule. This ability underlies gene cloning and all other aspects of recombinant DNA technology.

As previously described in methods the insertion of the various fragments into the pJQ200sk plasmid was sequential and before proceeding to next step. Correct fragment insertion was always confirmed by restriction analysis. Fig. 23 depicts an agarose gel electrophoresis of the restriction analysis of the last step of the construction. Lanes 2, 4 and 5 are positive samples with bands displaying the expected molecular weights (~6400 and ~2400 bp). The sample in lane 3 is negative. The higher molecular weight bands in lanes 2, 4 and 5 are uncompleted digested plasmid DNA. This can be due to several situations namely contaminants left by minipreps that might partially inhibit the activity of the restriction endonuclease and supercoiled plasmid DNA, which is generally more difficult to digest than linear DNA excess of plasmid DNA, or even excess undigested plasmid DNA.

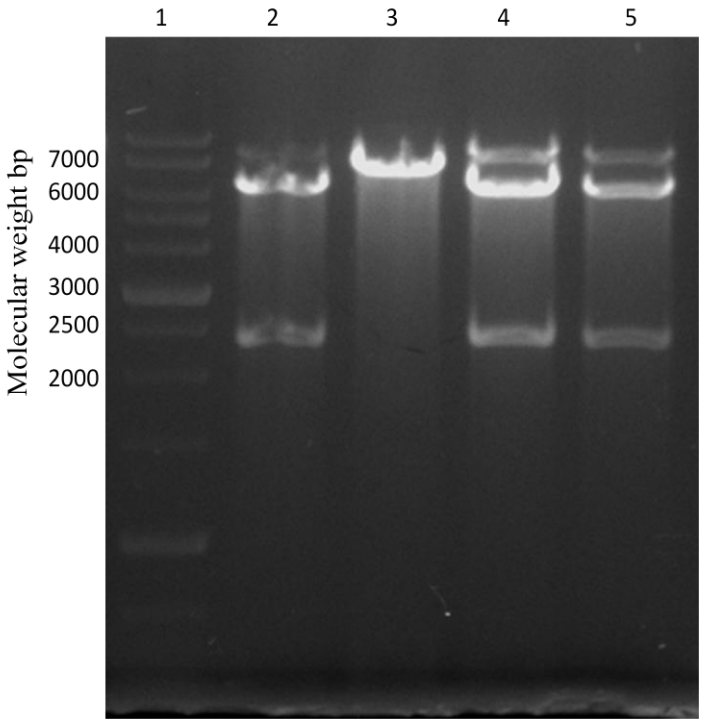


Figure 23 –Agarose gel electrophoresis 1% (ethidium bromide stain) following plasmid extraction and restriction analysis (NdeI) of four samples of the last step of the construction (MakF+PmyctetO+KanR2+Psmyc+TreSF). Lane 1: molecular weight marker (1Kb DNA ladder Bioron) Lanes 2, 4 and 5: positive samples (bands displaying 6400 and 2400 bp) Lane 3: negative sample

Chapter IV Conclusion

In order to examine specific functions of the Maltokinase the protein had to be purified. Protein purification is based on the separation of the protein of interest of contaminant proteins. This can be a time-consuming process. The purification of the recombinant His-tagged Mak was made less laborious by the modification of the sodium phosphate buffer pH. Subsequently several substrates were tested as phosphoryl group acceptors. As multidrug resistant *Mycobacterium* strains pose a serious obstacle to the control of tuberculosis and atypical infections and given that the Mak had an high sequence homology to aminoglycoside phosphotransferases it was tested if the enzyme could confer aminoglycoside resistance by phosphorylating aminoglycosides antibiotics. Beyond the aminoglycoside antibiotics disaccharides and trisaccharides were also tested as phosphate acceptors however no activity was detected, except for maltose, implicating that in *M. smegmatis* the role of phosphate acceptor might be specific to maltose. It is then extremely unlikely that the protein could render aminoglycoside resistance to bacteria.

RT-PCR is an exceptionally sensitive and useful quantification method and extremely adequate to analyze the transcriptional responses of the *mak* gene in different nutritional conditions, as proposed. Nevertheless, for accurate quantitation careful experimental design and validation are critical. The optimization of an RT-PCR experiment is time-consuming but absolutely essential in order to obtain accurate reproducible results.

Gene expression systems are valuable tools for the characterization of genes. The construction described here will consent the conditional expression of genes essential for mycobacterial growth and in doing so allow a better understanding of their involvement in mycobacterial metabolism.

In regard to the RT-PCR experiment and the conditional mutants the lack of time was the major issue, preventing us from reaching our intended objectives. However, if pursued, this broad spectrum approach can add to our knowledge of this enzyme's physiological role, its response to diverse conditions and also of its integration in the general mycobacterial metabolism.

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Annexes

1. Transformation

1.1. Preparation of Competent Cells

1.1.1. SOB Medium

Reagent	Amount per litter	Final concentration
Tryptone	20 g	2%
Yeast extract	5 g	0.5%
NaCl	0.5 g	0.05%
KCl	186 mg	2.5 mM
$MgCl_2$ 2 M	2.5 mL	5 mM

The pH was adjusted to 7. The solution was autoclaved and $MgCl_2$ was added only after cooling down the solution.

1.1.2. RF1 Solution

Reagent	Amount utilized	Final concentration
RbCl	3.02 g	100 mM
CH_3CO_2K	7.36 g	30 mM
$CaCl_2$	0.32 g	10 mM
$MnCl_2$	1.19 g	50 mM
Glycerol	0.05 g	15%

The reagents were added in sequential order to avoid precipitation. The pH was adjusted to 5.8 exactly, with acetic acid, once again to avoid precipitation of the reagents. Water was added to a total volume of 250 mL and the solution was filter sterilized.

1.1.3. RF2 Solution

Reagent	Amount utilized	Final concentration
MOPS	0.10 g	10 mM
RbCl	0.06 g	10 mM
$CaCl_2$	0.48 g	75 mM
Glycerol	0.01 g	15%

The solution's pH was adjusted to 6.8-7. Water was added to a final volume of 50 mL and the solution was filter sterilized.

Cells were grown in SOB medium supplemented with $MgCl_2$. Cells were harvested by centrifugation at $O.D._{610}$=0.3-0.4, 3000 r.p.m., 15 min, at 4ºC, following a 15 min incubation on ice. Cells pellet was ressuspended in 8 mL RF1 solution and incubated 15 min on ice. The suspension was once more centrifuged; the cell pellet was ressuspended in 2 mL of RF2 solution and incubated 15 min on ice. 100 µL aliquots were frozen at -80ºC.

1.1.4. 10x Stock Salt Solution

Reagent	Amount utilized	Final concentration
KCl	0.18 g	240 mM
$MgCl_2$	2 g	210 mM
$MgSO_4$	2.46 g	200 mM
Glucose filter sterilized	2 g	111 mM

MQ water was added to a final volume of 100 mL.

1.2. Transformation

The transforming DNA (10 µL of ligation) was added to the competent cells. The tubes were swirled gently to mix their contents and stored on ice for 20 minutes. The tubes ere then transferred for exactly 45 seconds to a preheated 42ºC water bath and .rapidly cooled on ice for 1-2 minutes. 500 µL of LB medium, with 10% salt solution, was added to the cells. The cultures were incubated at 37ºC for 45 minutes, to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. The appropriate volume (up to 200 µL per 90 mm plate) of transformed competent cells was transferred onto agar LB medium containing the suitable antibiotic (when working with pGEMT cloning vector 100 µl of each transformation culture was plated onto LB/ampicillin/IPTG/X-Gal plates). Plates were incubated at 37°C.

2. Agarose Gel Electrophoresis

2.1. TAE 50x

Reagent	Amount per liter	Final concentration
Tris base	242 g	2 M
Acetic acid	57.1 mL	2 M
Aqueous solution of EDTA 0.5 mM pH8	100 mL	0.05 M

Tris (Merck) was dissolved in the aqueous solution of EDTA (Merck) while heating the mix. Acetic acid was added (José M. Vaz Pereira). pH was adjusted to 8 with NaOH 5 M. H_2O was added to a final volume of 1 L.

2.2. Agarose Gel (1% or 2%)

Reagent	Amount utilized	Final concentration
Agarose	1.5 g	1%
	3 g	2%
TAE1x	150 mL	1x

Agarose (Molecular Biology Grade Bioron for electrophoresis) was dissolved in TAE buffer 1x, heated to boiling point and swirled to facilitate dissolution. The mix was cooled down to 60°C, ethidium bromide (10 mg/mL stock solution) was then added to a final concentration of 0.5 µg/ml (8 µL). The solution was stirred to disperse the ethidium bromide, poured slowly into the gel rack, the combs were inserted and the gel was left to gelate. The gel was run at 4v/cm for 30min, for 1% gel, and for 45 min for 2% gel.

As described in Sambrook & Russel (2001) chapter 5, protocol 1 and 2.

3. 1.2% Formaldehyde Agarose Gel Electrophoresis

3.1. 10x Formaldehyde Agarose Gel Buffer

Reagent	Amount utilized	Final concentration
MOPS (free acid)	20.93 g	200 mM
Sodium acetate	2.06 g	50 mM
EDTA	1.86 g	10 mM

pH was adjusted to 7.0 with NaOH.

3.2. 1x Formaldehyde Agarose Gel Running Buffer

Reagent	Amount utilized	Final concentration
10x Formaldehyde Agarose gel buffer	50 mL	1x
37% (12.3 M) formaldehyde	10 mL	123 mM
RNase-free water	440 mL	-

3.3. 5X RNA Loading Buffer

Reagent	Amount utilized	Final concentration
Saturated aqueous bromophenol blue solution	16 µL	-
EDTA 500 mM, pH 8.0	80 µL	4 mM
Formaldehyde 37% (12.3 M)	720 µL	880 mM
Glycerol 100%	2 mL	20%
Formamide	3084 µL	30.84%
10x Formaldehyde Agarose gel buffer	4 mL	4x

RNase-free water to was added to a final volume of 10 mL

0.6 g of agarose were dissolved in 5 mL of 10x Formaldehyde Agarose Gel Buffer and 45 mL of RNase free water, the solution was heated to boiling point and swirled to facilitate dissolution. The mix was cooled down to 60°C. 1.8 ml of 37% formaldehyde and ethidium bromide (10 mg/mL stock solution) to final concentration of 0.5 µg/ml (3 µL) were added in a fume hood. The solution was stirred and poured slowly into the gel rack, the comb was inserted and the gel was left to gelate. Prior to running the gel it was equilibrated in 1x Formaldehyde Agarose gel running buffer for 30 min.

4. Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

4.1. Resolving Gel (12%)

Reagent	Amount utilized	Final concentration
Acrylamide/Bis, 37.5:1 mixture (30%T, 2.67% C) (Biorad)	2 mL	12%
H_2O	1.7 mL	-
1.5 M Tris-HCl, pH 8.8	1.25 mL	0.4 M
SDS 10% (w/v)	50 µL	0.1%
Just before pouring add catalysts		
TEMED	**5 µl**	**ND***
Ammonium Persulfate (APS) 10% (w/v)	30 µl	0.06%

Swirl gently to initiate polymerization

*Not determined

4.2. Stacking Gel (4%)

Reagent	Amount utilized	Final concentration
Acrylamide/Bis, 37.5:1 mixture (30%T, 2.67% C) (Biorad)	325 µL	4%
H_2O	1.525 mL	-
1.5 M Tris-HCl, pH 8.8	625 µL	0.4 M
SDS 10% (w/v)	25 µL	0,1%
Just before pouring add catalysts:		
TEMED	**5 µl**	**ND***
Ammonium Persulfate (APS) 10% (w/v)	30 µl	0,12%

Swirl gently to initiate polymerization

4.3. Sample Buffer (SDS Reducing Buffer)

Reagent	Amount utilized	Final concentration
H_2O	3.55 mL	-
0.5 M Tris-HCl, pH 6.8	1.25 mL	0.06 M
Glycerol	2.5 mL	0.26%
SDS 10% (w/v)	2.0 mL	2.1%
Bromophenol blue 0.5%(w/v)	0.2 mL	0.01%

4.4. 10x Running Buffer, pH 8.3

Reagent	Amount per liter	Final concentration
Tris base	30.3 g	0.25 M
Glycine	144.0 g	1.92 M
SDS	10.0 g	10%

The reagents were dissolved and the total volume brought up to 1 L with water. This solution pH cannot be adjusted with acid or base.

4.5. Coomassie Stain

Reagent	Amount per litter	Final concentration
Coomassie R-250	1 g	1%
Glacial acetic acid	100 mL	10% (v)
Methanol	400 mL	40% (v)

100 mL of glacial acetic acid were added to 500 mL of MQ H_2O. Subsequently 500 mL of methanol were added. Finally 1 g of Coomassie dye was added the mixture was swirled and then filtered to remove particulates.

4.6. Destaining Solution

Reagent	Amount per litter	Final concentation
Methanol	250 mL	25% (v)
Glacial acetic acid	75 mL	7.5% (v)

250 mL of glacial acetic acid were added to 675 mL of MQ H_2O and then 500 mL of methanol were added to the mixture.

The glass cassette and casting stand were assembled. Resolving gel solution was prepared by combining all reagents and poured into the glass cassette 1 cm below the comb teeth level. The solution was overlaid with buthanol and left to polymerize for 30 min. The gel surface was completely rinsed with water and traces of water were removed with a piece of paper. The resolving gel solution was prepared by combining all reagents. The solution was poured into the cassette, the combs were inserted and the gel was left to polymerize for 30 min. After the stacking gel polymerization, the combs were removed; the tank was assembled, loaded and run at 200 V (constant V) for 45 min.

Samples were prepared by adding an equal volume of 2×SDS sample buffer with 5% β-Mercaptoethanol to the sample (still on ice) and were then incubated at 95°C for 5 min.